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(54) Title: METHOD FOR CREATING POLYNUCLEOTIDE AND POLYPEPTIDE SEQUENCES

(57) Abstract

The invention provides methods for evolving a polynucleotide toward acquisition of a desired property. Such methods entail incubating a population of parental polynucleotide variants under conditions to generate annealed polynucleotides comprising heteroduplexes. The heteroduplexes are then exposed to a cellular DNA repair system to convert the heteroduplexes to parental polynucleotide variants or recombined polynucleotide variants. The resulting polynucleotides are then screened or selected for the desired property.

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METHOD FOR CREATING POLYNUCLEOTIDE AND POLYPEPTIDE SEQUENCES

CROSS-REFERENCES TO RELATED APPLICATIONS

This application derives priority from USSN 60/067908, filed December 8, 1997, which is incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

The invention resides in the technical field of genetics, and more specifically, forced molecular evolution of polynucleotides to acquire desired properties.

BACKGROUND

A variety of approaches, including rational design and directed evolution, have been used to optimize protein functions (1, 2). The choice of approach for a given optimization problem depends, in part, on the degree of understanding of the relationships between sequence, structure and function. Rational redesign typically requires extensive knowledge of a structure-function relationship. Directed evolution requires little or no specific knowledge about structure-function relationship; rather, the essential features is a means to evaluate the function to be optimized. Directed evolution involves the generation of libraries of mutant molecules followed by selection or screening for the desired function. Gene products which show improvement with respect to the desired property or set of properties are identified by selection or screening. The gene(s) encoding those products can be subjected to further cycles of the process in order to accumulate beneficial mutations. This evolution can involve few or many generations, depending on how far one wishes to progress and the effects of mutations typically observed in each generation. Such approaches have been used to create novel functional nucleic acids (3, 4), peptides and other small molecules (3), antibodies (3), as well as enzymes and other proteins (5, 6, 7). These procedures are fairly tolerant to inaccuracies and noise in the function evaluation (7).

Several publications have discussed the role of gene recombination in directed evolution (see WO 97/07205, WO 98/42727, US 5807723, US 5,721,367, US 5,776,744 and WO 98/41645 US 5,811,238, WO 98/41622, WO 98/41623, and US 5,093,257).

A PCR-based group of recombination methods consists of DNA shuffling [5, 6], staggered extension process [89, 90] and random-priming recombination [87]. Such methods typically involve synthesis of significant amounts of DNA during assembly/recombination step and subsequent amplification of the final products and the efficiency of amplification decreases with gene size increase.

Yeast cells, which possess an active system for homologous recombination, have been used for *in vivo* recombination. Cells transformed with a vector and partially overlapping inserts efficiently join the inserts together in the regions of homology and restore a functional, covalently-closed plasmid [91]. This method does not require PCR amplification at any stage of recombination and therefore is free from the size considerations inherent in this method. However, the number of crossovers introduced in one recombination event is limited by the efficiency of transformation of one cell with multiple inserts. Other *in vivo* recombination methods entail recombination between two parental genes cloned on the same plasmid in a tandem orientation. One method relies on homologous recombination machinery of bacterial cells to produce chimeric genes [92]. A first gene in the tandem provides the N-terminal part of the target protein, and a second provides the C-terminal part. However, only one crossover can be generated by this approach. Another *in vivo* recombination method uses the same tandem organization of substrates in a vector [93]. Before transformation into *E. coli* cells, plasmids are linearized by endonuclease digestion between the parental sequences. Recombination is performed *in vivo* by the enzymes responsible for double-strand break repair. The ends of linear molecules are degraded by a 5'->3' exonuclease activity, followed by annealing of complementary single-strand 3' ends and restoration of the double-strand plasmid [94]. This method has similar advantages and disadvantages of tandem recombination on circular plasmid.

SUMMARY OF THE INVENTION

The invention provides methods for evolving a polynucleotide toward acquisition of a desired property. Such methods entail incubating a population of

parental polynucleotide variants under conditions to generate annealed polynucleotides comprises heteroduplexes. The heteroduplexes are then exposed to a cellular DNA repair system to convert the heteroduplexes to parental polynucleotide variants or recombined polynucleotide variants. The resulting polynucleotides are then screened or selected for the desired property.

In some methods, the heteroduplexes are exposed to a DNA repair system *in vitro*. A suitable repair system can be prepared in the form of cellular extracts.

In other methods, the products of annealing including heteroduplexes are introduced into host cells. The heteroduplexes are thus exposed to the host cells' DNA repair system in vivo.

In several methods, the introduction of annealed products into host cells selects for heteroduplexes relative to transformed cells comprising homoduplexes. Such can be achieved, for example, by providing a first polynucleotide variant as a component of a first vector, and a second polynucleotide variant is provided as a component of a second vector. The first and second vectors are converted to linearized forms in which the first and second polynucleotide variants occur at opposite ends. In the incubating step, single-stranded forms of the first linearized vector reanneal with each other to form linear first vector, single-stranded forms of the second linearized vector reanneal with each other to form linear second vector, and single-stranded linearized forms of the first and second vectors anneal with each to form a circular heteroduplex bearing a nick in each strand. Introduction of the products into cells thus selects for cirular heteroduplexes relative to the linear first and second vector. Optionally, in the above methods, the first and second vectors can be converted to linearized forms by PCR. Alternatively, the first and second vectors can be converted to linearized forms by digestion with first and second restriction enzymes.

In some methods, polynucleotide variants are provided in double stranded form and are converted to single stranded form before the annealing step. Optionally, such conversion is by conducting asymmetric amplification of the first and second double stranded polynucleotide variants to amplify a first strand of the first polynucleotide variant, and a second strand of the second polynucleotide variant. The first and second strands anneal in the incubating step to form a heteroduplex.

In some methods, a population of polynucleotides comprising first and second polynucleotides is provided in double stranded form, and the method further

comprises incorporating the first and second polynucleotides as components of first and second vectors, whereby the first and second polynucleotides occupy opposite ends of the first and second vectors. In the incubating step single-stranded forms of the first linearized vector reanneal with each other to form linear first vector, single-stranded forms of the second linearized vector reanneal with each other to form linear second vector, and single-stranded linearized forms of the first and second vectors anneal with each to form a circular heteroduplex bearing a nick in each strand. In the introducing step selects for transformed cells comprises the circular heteroduplexes relative to the linear first and second vector.

In some methods, the first and second polynucleotides are obtained from chromosomal DNA. In some methods, the polynucleotide variants encode variants of a polypeptide. In some methods, the population of polynucleotide variants comprises at least 20 variants. In some methods, the population of polynucleotide variants are at least 10 kb in length.

In some methods, the polynucleotide variants comprises natural variants. In other methods, the polynucleotide variants comprise variants generated by mutagenic PCR or cassette mutagenesis. In some methods, the host cells into which heteroduplexes are introduced are bacterial cells. In some methods, the population of variant polynucleotide variants comprises at least 5 polynucleotides having at least 90% sequence identity with one another.

Some methods further comprise a step of at least partially demethylating variant polynucleotides. Demethylation can be achieved by PCR amplification or by passaging variants through methylation-deficient host cells.

Some methods include a further step of sealing one or more nicks in heteroduplex molecules before exposing the heteroduplexes to a DNA repair system. Nicks can be sealed by treatment with DNA ligase.

Some methods further comprise a step of isolating a screened recombinant polynucleotide ariant. In some methods, the polynucleotide variant is screened to produce a recombinant protein or a secondary metabolite whose production is catalyzed thereby.

In some methods, the recombinant protein or secondary metabolite is formulated with a carrier to form a pharmaceutical composition.

In some methods, the polynucleotide variants encode enzymes selected from the group consisting of proteases, lipases, amylases, cutinases, cellulases, amylases, oxidases, peroxidases and phytases. In other methods, the polynucleotide variants encode a polypeptide selected from the group consisting of insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erthropoietin, luteinizing hormone, chorionic gonadotropin, hyperthalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon, thrombopoietic (TPO), and prolactin.

In some methods, each polynucleotide in the population of variant polynucleotides encodes a plurality of enzymes forming a metabolic pathway.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the process of heteroduplex formation using polymerase chain reaction (PCR) with one set of primers for each different sequence to amplify the target sequence and vector.

Figure 2 illustrates the process of heteroduplex formation using restriction enzymes to linearize the target sequences and vector.

Figure 3 illustrates a process of heteroduplex formation using asymmetric or single primer polymerase chain reaction (PCR) with one set of primers for each different sequence to amplify the target sequence and vector.

Figure 4 illustrates heteroduplex recombination using unique restriction enzymes (X and Y) to remove the homoduplexes.

Figure 5 shows the amino acid sequences of the FlaA from *R. lupini* (SEQ ID NO: 1) and *R. meliloti* (SEQ ID NO:2).

Figures 6A and 6B show the locations of the unique restriction sites utilized to linearize pRL20 and pRM40.

Figures 7A, B, C and D show the DNA sequences of four mosaic *flaA* genes created by *in vitro* heteroduplex formation followed by *in vivo* repair ((a) is SEQ ID NO:3, (b) is SEQ ID NO:4, (c) is SEQ ID NO:5 and (d) is SEQ ID NO:6).

Figure 8 illustrates how the heteroduplex repair process created mosaic *flaA* genes containing sequence information from both parent genes.

Figure 9 shows the physical maps of *Actinoplanes utahensis* ECB deacylase mutants with enhanced specific activity ((a) is pM7-2 for Mutant 7-2, and (b) is pM16 for Mutant 16).

Figure 10 illustrates the process used for Example 2 to recombine mutations in Mutant 7-2 and Mutant 16 to yield ECB deacylase recombinant with more enhanced specific activity.

Figure 11 shows specific activities of wild-type ECB deacylase and improved mutants Mutant 7-2, Mutant 16 and recombined Mutant 15.

Figure 12 shows positions of DNA base changes and amino acid substitutions in recombined ECB deacylase Mutant 15 with respect to parental sequences of Mutant 7-2 and Mutant 16.

Figures 13 A, B, C, D and E show the DNA sequence of *A.utahensis* ECB deacylase gene mutant M-15 genes created by *in vitro* heteroduplex formation followed by *in vivo* repair (SEQ ID NO:7).

Figure 14 illustrates the process used for Example 3 to recombine mutations in RC1 and RC2 to yield thermostable subtilisin E.

Figure 15 illustrates the sequences of RC1 and RC2 and the ten clones picked randomly from the transformants of the reaction products of duplex formation as described in Example 3. The x's correspond to base positions that differ between RC1 and RC2. The mutation at 995 corresponds to amino acid substitution at 181, while that at 1107 corresponds to an amino acid substitution at 218 in the subtilisin protein sequence.

Figure 16 shows the results of screening 400 clones from the library created by heteroduplex formation and repair for initial activity (A_i) and residual activity (A_r). The ratio A_i/A_r was used to estimate the enzymes' thermostability. Data from active variants are sorted and plotted in descending order. Approximately 12.9% of the clones exhibit a phenotype corresponding to the double mutant containing both the N181D and the N218S mutations.

DEFINITIONS

Screening is, in general, a two-step process in which one first physically separates the cells and then determines which cells do and do not possess a desired property. Selection is a form of screening in which identification and physical separation are achieved simultaneously by expression of a selection marker, which, in some genetic

circumstances, allows cells expressing the marker to survive while other cells die (or vice versa). Exemplary screening members include luciferase, βgalactosidase and green fluorescent protein. Selection markers include drug and toxin resistance genes. Although spontaneous selection can and does occur in the course of natural evolution, in the present methods selection is performed by man.

An exogenous DNA segment is one foreign (or heterologous) to the cell or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

The term gene is used broadly to refer to any segment of DNA associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. Genes also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins.

The term "wild-type" means that the nucleic acid fragment does not comprise any mutations. A "wild-type" protein means that the protein will be active at a level of activity found in nature and typically will comprise the amino acid sequence found in nature. In an aspect, the term "wild type" or "parental sequence" can indicate a starting or reference sequence prior to a manipulation of the invention.

"Substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual macromolecular species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species.

Percentage sequence identity is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of

positions in the window of comparison. Optimal alignment of sequences for aligning a comparison window can be conducted by computerized implementations of algorithms GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI.

The term naturally-occurring is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring. Generally, the term naturally-occurring refers to an object as present in a non-pathological (undiseased) individual, such as would be typical for the species.

A nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it increases the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

A specific binding affinity between, for example, a ligand and a receptor, means a binding affinity of at least $1 \times 10^6\ M^{-1}$.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human CD4 gene is the cognate gene to the mouse CD4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition.

The term "heteroduplex" refers to hybrid DNA generated by base pairing between complementary single strands derived from the different parental duplex molecules, whereas the term "homoduplex" refers to double-stranded DNA generated by base pairing between complementary single strands derived from the same parental duplex molecules.

The term "nick" in duplex DNA refers to the absence of a phosphodiester bond between two adjacent nucleotides on one strand. The term "gap" in duplex DNA refers to an absence of one or more nucleotides in one strand of the duplex. The term "loop" in duplex DNA refers to one or more unpaired nucleotides in one strand.

A mutant or variant sequence is a sequence showing substantial variation from a wild type or reference sequence that differs from the wild type or reference sequence at one or more positions.

DETAILED DESCRIPTION

I. General

The invention provides methods of evolving a polynucleotide toward acquisition of a desired property. The substrates for the method are a population of at least two polynucleotide variant sequences that contain regions of similarity with each other but, which also have point(s) or regions of divergence. The substrates are annealed *in vitro* at the regions of similarity. Annealing can regenerate initial substrates or can form heteroduplexes, in which component strands originate from different parents. The products of annealing are exposed to enzymes of a DNA repair, and optionally a replication system, that repairs unmatched pairings. Exposure can be *in vivo* as when annealed products are transformed into host cells and exposed to the hosts DNA repair system. Alternatively, exposure can be *in vitro*, as when annealed products are exposed to cellular extracts containing functional DNA repair systems. Exposure of heteroduplexes to a DNA repair system results in DNA repair at bulges in the heteroduplexes due to DNA mismatching. The repair process differs from homologous recombination in promoting nonreciprocal exchange of diversity between strands. The DNA repair process is typically effected on both component strands of a heteroduplex molecule and at any particular mismatch is typically random as to which strand is repaired. The resulting population can thus contain recombinant polynucleotides encompassing an essentially random reassortment of points of divergence between parental strands. The population of recombinant polynucleotides is then screened for acquisition of a desired property. The property can be a property of the polynucleotide *per se*, such as capacity of a DNA molecule to bind to a protein or can be a property of an expression product thereof, such as mRNA or a protein.

II. Substrates For Shuffling

The substrates for shuffling are variants of a reference polynucleotide that show some region(s) of similarity with the reference and other region(s) or point(s) of divergence. Regions of similarity should be sufficient to support annealing of polynucleotides such that stable heteroduplexes can be formed. Variants forms often show substantial sequence identity with each other (e.g., at least 50%, 75%, 90% or 99%). There should be at least sufficient diversity between substrates that recombination can generate more diverse products than there are starting materials. Thus, there must be at least two substrates differing in at least two positions. The degree of diversity depends on the length of the substrate being recombined and the extent of the functional change to be evolved. Diversity at between 0.1-25% of positions is typical. Recombination of mutations from very closely related genes or even whole sections of sequences from more distantly related genes or sets of genes can enhance the rate of evolution and the acquisition of desirable new properties. Recombination to create chimeric or mosaic genes can be useful in order to combine desirable features of two or more parents into a single gene or set of genes, or to create novel functional features not found in the parents. The number of different substrates to be combined can vary widely in size from two to 10, 100, 1000, to more than 10^5, 10^7, or 10^9 members.

The initial small population of the specific nucleic acid sequences having mutations may be created by a number of different methods. Mutations may be created by error-prone PCR. Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. Alternatively, mutations can be introduced into the template polynucleotide by oligonucleotide-directed mutagenesis. In oligonucleotide-directed mutagenesis, a short sequence of the polynucleotide is removed from the polynucleotide using restriction enzyme digestion and is replaced with a synthetic polynucleotide in which various bases have been altered from the original sequence. The polynucleotide sequence can also be altered by chemical mutagenesis. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid. Other agents which are analogues of nucleotide precursors include nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. Generally, these agents are added to the PCR reaction in place of the nucleotide precursor thereby mutating the sequence. Intercalating agents such as proflavine,

acriflavine, quinacrine and the like can also be used. Random mutagenesis of the polynucleotide sequence can also be achieved by irradiation with X-rays or ultraviolet light. Generally, plasmid DNA or DNA fragments so mutagenized are introduced into E. coli and propagated as a pool or library of mutant plasmids.

Alternatively the small mixed population of specific nucleic acids can be found in nature in the form of different alleles of the same gene or the same gene from different related species (i.e., cognate genes). Alternatively, substrates can be related but nonallelic genes, such as the immunoglobulin genes. Diversity can also be the result of previous recombination or shuffling. Diversity can also result from resynthesizing genes encoding natural proteins with alternative codon usage.

The starting substrates encode variant forms of sequences to be evolved. In some methods, the substrates encode variant forms of a protein for which evolution of a new or modified property is desired. In other methods, the substrates can encode variant forms of a plurality of genes constituting a multigene pathway. In such methods, variation can occur in one or any number of the component genes. In other methods, substrates can contain variants segments to be evolved as DNA or RNA binding sequences. In methods, in which starting substrates containing coding sequences, any essential regulatory sequences, such as a promoter and polyadenylation sequence, required for expression may also be present as a component of the substrate. Alternatively, such regulatory sequences can be provided as components of vectors used for cloning the substrates.

The starting substrates can vary in length from about 50, 250, 1000, 10,000, 100,000, 10^6 or more bases. The starting substrates can be provided in double- or single-stranded form. The starting substrates can be DNA or RNA and analogs thereof. If DNA, the starting substrates can be genomic or cDNA. If the substrates are RNA, the substrates are typically reverse-transcribed to cDNA before heteroduplex formation. Substrates can be provided as cloned fragments, chemically synthesized fragments or PCR amplification products. Substrates can derive from chromosomal, plasmid or viral sources. In some methods, substrates are provided in concatemeric form.

III. Procedures for Generating Heteroduplexes

Heteroduplexes are generated from double stranded DNA substrates, by denaturing the DNA substrates and incubating under annealing conditions. Hybridization

conditions for heteroduplex formation are sequence-dependent and are different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, hybridization conditions are selected to be about 25°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium.

Exemplary conditions for denaturation and renaturation of double stranded substrates are as follows. Equimolar concentrations (~ 1.0 - 5.0 nM) of the substrates are mixed in 1 x SSPE buffer (180 mM NaCl, 1.0 mM EDTA, 10 mM NaH_2PO_4, pH 7.4) After heating at 96°C for 10 minutes, the reaction mixture is immediately cooled at 0°C for 5 minutes; The mixture is then incubated at 68°C for 2-6 hr. Denaturation and reannealing can also be carried out by the addition and removal of a denaturant such as NaOH. The process is the same for single stranded DNA substrates, except that the denaturing step may be omitted for short sequences.

By appropriate design of substrates for heteroduplex formation, it is possible to achieve selection for heteroduplexes relative to reformed parental homoduplexes. Homoduplexes merely reconstruct parental substrates and effectively dilute recombinant products in subsequent screening steps. In general, selection is achieved by designing substrates such that heteroduplexes are formed in open-circles, whereas homoduplexes are formed as linear molecules. A subsequent transformation step results in substantial enrichment (e.g., 100-fold) for the circular heteroduplexes.

Figure 1 shows a method in which two substrate sequences in separate vectors are PCR-amplified using two different sets of primers (P1, P2 and P3, P4). Typically, first and second substrates are inserted into separate copies of the same vector. The two different pairs of primers initiate amplification at different points on the two vectors. Fig. 1 shows an arrangement in which the P1/P2 primer pairs initiates amplification at one of the two boundaries of the vector with the substrate and the P1/P2 primer pair initiates replication at the other boundary in a second vector. The two primers in each primer pair prime amplification in opposite directions around a circular plasmid. The amplification products generated by this amplification are double-stranded linearized vector molecules in which the first and second substrates occur at opposite ends of the

vector. The amplification products are mixed, denatured and annealed. Mixing and denaturation can be performed in either order. Reannealing generates two linear homoduplexes, and an open circular heteroduplex containing one nick in each strand, at the initiation point of PCR amplification. Introduction of the amplification products into host cells selects for the heteroduplexes relative to the homoduplexes because the former transform much more efficiently than the latter.

It is not essential in the above scheme that amplification is initiated at the interface between substrate and the rest of the vector. Rather, amplification can be initiated at any points on two vectors bearing substrates provided that the amplification is initiated at different points between the vectors. In the general case, such amplification generates two linearized vectors in which the first and second substrates respectively occupy different positions relative to the remainder of the vector. Denaturation and reannealing generator heteroduplexes similar to that shown in Fig. 1, except that the nicks occur within the vector component rather than at the interface between plasmid and substrate. Initiation of amplification outside the substrate component of a vector has the advantage that it is not necessary to design primers specific for the substrate borne by the vector.

Although Fig. 1 is exemplified for two substrates, the above scheme can be extended to any number of substrates. For example, an initial population of vector bearing substrates can be divided into two pools. One pool is PCR-amplified from one set of primers, and the other pool from another. The amplification products are denatured and annealed as before. Heteroduplexes can form containing one strand from any substrate in the first pool and one strand from any substrate in the second pool. Alternatively, three or more substrates cloned into multiple copies of a vector can be subjected to amplification with amplification in each vector starting at a different point. For each substrate, this process generates amplification products varying in how flanking vector DNA is divided on the two sides of the substrate. For example, one amplification product might have most of the vector on one side of the substrate, another amplification product might have most of the vector on the other side of the substrate, and a further amplification product might have an equal division of vector sequence flanking the substrate. In the subsequent annealing step, a strand of substrate can form a circular heteroduplex with a strand of any other substrate, but strands of the same substrate can only reanneal with each other to form a linear homoduplex. In a still further variation,

multiple substrates can be performed by performing multiple iterations of the scheme in Fig. 1. After the first iteration, recombinant polynucleotides in a vector, undergo heteroduplex formation with a third substrate incorporated into a further copy of the vector. The vector bearing the recombinant polynucleotides and the vector bearing the third substrate are separately PCR amplified from different primer pairs. The amplification products are then denatured and annealed. The process can be repeated further times to allow recombination with further substrates.

An alternative scheme for heteroduplex formation is shown in Fig. 2. Here, first and second substrates are incorporated into separate copies of a vector. The two copies are then respectively digested with different restriction enzymes. Fig. 2 shows an arrangement in which, the restriction enzymes cut at opposite boundaries between substrates and vector, but all that is necessary is to use two different restriction enzymes that cut at different places. Digestion generates linearized first and second vector bearing first and second substrates, the first and second substrates occupying different positions relative to the remaining vector sequences. Denaturation and reannealing generates open circular heteroduplexes and linear homoduplexes. The scheme can be extended to recombination between more than two substrates using analogous strategies to those described with respect to Fig. 1. In one variation, two pools of substrates are formed, and each is separately cloned into vector. The two pools are then cute with different enzymes, and annealing proceeds as for two substrates. In another variation, three or more substrates can be cloned into three or more copies of vector, and the three or more result molecules cut with three or more enzymes, cutting at three or more sites. This generates three different linearized vector forms differing in the division of vector sequences flanking the substrate moiety in the vectors. Alternatively, any number of substrates can be recombined pairwise in an iterative fashion with products of one round of recombination annealing with a fresh substrate in each round.

In a further variation, heteroduplexes can be formed from substrates molecules in vector-free form, and the heteroduplexes subsequently cloned into vectors. Such can be achieved by asymmetric amplification of first and second substrates as shown in Fig. 3. Asymmetric or single primer PCR amplifies only one strand of a duplex. By appropriate selection of primers, opposite strands can be amplified from two different substrates. On reannealing amplification products, heteroduplexes are formed from opposite strands of the two substrates. Because only one strand is amplified from each

substrate, reannealing does not reform homoduplexes (other than for small quantities of unamplified substrate). The process can be extended to allow recombination of any number of substrates using analogous strategies to those described with respect to Fig. 1. For example, substrates can be divided into two pools, and each pool subject to the same asymmetric amplification, such that amplification products of one pool can only anneal with amplification products of the other pool , and not with each other. Alternatively, shuffling can proceed pairwise in an iterative manner, in which recombinants formed from heteroduplexes of first and second substrates, are subsequently subjected to heteroduplex formation with a third substrate. Point mutations can also be introduced at a desired level during PCR amplification.

Fig. 4 shows another approach of selecting for heteroduplexes relative to homoduplexes. First and second substrates are isolated by PCR amplification from separate vectors. The substrates are denatured and allowed to anneal forming both heteroduplexes and reconstructed homoduplexes. The products of annealing are digested with restriction enzymes X and Y. X has a site in the first substrate but not the second substrate, and vice versa for Y. Enzyme X cuts reconstructed homoduplex from the first substrate and enzyme Y cuts reconstructed homoduplex from the second substrate. Neither enzyme cuts heteroduplexes. Heteroduplexes can effectively be separated from restriction fragments of homoduplexes by further cleavage with enzymes A and B having sites proximate to the ends of both the first and second substrates, and ligation of the products into vector having cohesive ends compatible with ends resulting from digestion with A and B. Only heteroduplexes cut with A and B can ligate with the vector. Alternatively, heteroduplexes can be separated from restriction fragments of homoduplexes by size selection on gels. The above process can be generalized to N substrates by cleaving the mixture of heteroduplexes and homoduplexes with N enzymes, each one of which cuts a different substrate and no other substrate. Heteroduplexes can be formed by directional cloning. Two substrates for heteroduplex formation can be obtained by PCR amplification of chromosomal DNA and joined to opposite ends of a linear vector. Directional cloning can be achieved by digesting the vector with two different enzymes, and digesting or adapting first and second substrates to be respectively compatible with cohesive ends of only of the two enzymes used to cut the vector. The first and second substrates can thus be ligated at opposite ends of a linearized vector fragment. This scheme can be extended to any number of substrates by using principles

analogous to those described for Fig. 1. For example, substrates can be divided into two pools before ligation to the vector. Alternatively, recombinant products formed by heteroduplex formation of first and second substrates, can subsequently undergo heteroduplex formation with a third substrate.

IV. Vectors and Transformation

In general, substrates are incorporated into vectors either before or after the heteroduplex formation step. A variety of cloning vectors typically used in genetic engineering are suitable.

The vectors containing the DNA segments of interest can be transferred into the host cell by standard methods, depending on the type of cellular host. For example, calcium chloride transformation is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment. Lipofection, or electroporation may be used for other cellular hosts. Other methods used to transform mammalian cells include the use of Polybrene, protoplast fusion, liposomes, electroporation, and microinjection, and biolisitics (see, generally, Sambrook et al., supra). Viral vectors can also be packaged *in vitro* and introduced by infection. The choice of vector depends on the host cells. In general, a suitable vector has an origin of replication recognized in the desired host cell, a selection maker capable of being expressed in the intended host cells and/or regulatory sequences to support expression of genes within substrates being shuffled.

V. Types of Host Cells

In general any type of cells supporting DNA repair and replication of heteroduplexes introduced into the cells can be used. Cells of particular interest are the standard cell types commonly used in genetic engineering, such as bacteria, particularly, *E. coli (16, 17)*. Suitable *E. coli* strains include *E. coli mutS, mutL, dam*$^-$, and/or *recA*$^+$, *E.coli* XL-10-Gold ([*Tet*^{r}*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte] [F' proAB lacI*^{q}*ZΔM15 Tn10 (Tet*^{r}*) Amy Cam*^{r}*]*), *E. coli* ES1301 *mutS* [Genotype: *lacZ53, mutS201::Tn5, thyA36, rha-5, metB1, deoC, IN(rrnD-rrnE)*] (20, 24, 28-42). Preferred E. coli strains are *E.coli* SCS110 [Genotype: *rpsl. (Str*^{r}*), thr, leu, enda, thi-1, lacy, galk, galt, ara tona, tsx, dam, dcm, supE44, Δ(lac-proAB), [F, traD36, proA*$^-$*B*$^-$*lacI*^{q}*ZΔM15*], which have normal cellular mismatch repair

systems (17). This strain type repairs mismatches and unmatches in the heteroduplex with little strand-specific preference. Further, because this strain is *dam*⁻ and *dcm*⁻, plasmid isolated from the strain is unmethylated and therefore particularly amenable for further rounds of DNA duplex formation/mismatch repair (see below). Other suitable bacterial cells include gram-negative and gram-positive, such as *Bacillus, Pseudomonas,* and *Salmonella.*

Eukaryotic organisms are also able to carry out mismatch repair (43-48). Mismatch repair systems in both prokaryotes and eukaryotes are thought to play an important role in the maintenance of genetic fidelity during DNA replication. Some of the genes that play important roles in mismatch repair in prokaryotes, particularly *mutS* and *mutL*, have homologs in eukaryotes, in the outcome of genetic recombinations, and in genome stability. Wild-type or mutant *S. cerevisiae* has been shown to carry out mismatch repair of heteroduplexes (49-56), as have COS-1 monkey cells (57). Preferred strains of yeast are Picchia and Saccharomyces. Mammalian cells have been shown to have the capacity to repair G-T to G-C base pairs by a short-patch mechanism (38, 58-63). Mammalian cells (e.g., mouse, hamster, primate, human), both cell lines and primary cultures can also be used. Such cells include stem cells, including embryonic stem cells, zygotes, fibroblasts, lymphocytes, Chinese hamster ovary (CHO), mouse fibroblasts (NIH3T3), kidney, liver, muscle, and skin cells. Other eucaryotic cells of interest include plant cells, such as maize, rice, wheat, cotton, soybean, sugarcane, tobacco, and arabidopsis; fish, algae, fungi (aspergillus, podospora, neurospora), insect (e.g., baculo lepidoptera) (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., (1987), which is incorporated herein by reference).

In vivo repair occurs in a wide variety of prokaryotic and eukaryotic cells. Use of mammalian cells is advantage in certain application in which substrates encode polypeptides that are expressed only in mammalian cells or which are intended for use in mammalian cells. However, bacterial and yeast cells are advantageous for screening large libraries due to the higher transformation frequencies attainable in these strains.

V. In Vitro DNA Repair Systems

As an alternative to introducing annealed products into host cells, annealed products can be exposed a DNA repair system *in vitro*. The DNA repair system can be obtained as extracts from repair-competent *E. coli*, yeast or any other cells (64-67).

Repair-competent cells are lysed in appropriate buffer and supplemented with nucleotides. DNA is incubated in this cell extract and transformed into competent cells for replication.

VI. Screening and Selection

After introduction of annealed products into host cells, the host cells are typically cultured to allow repair and replication to occur and optionally, for genes encoded by polynucleotides to be expressed. The recombinant polynucleotides can be subject to further rounds of recombination using the heteroduplex procedures described above, or other shuffling methods described below. However, whether after one cycle of recombination or several, recombinant polynucleotides are subjected to screening or selection for a desired property. In some instances, screening or selection in performed in the same host cells that are used for DNA repair. In other instances, recombinant polynucleotides, their expression products or secondary metabolites produced by the expression products are isolated from such cells and screened in vitro. In other instances, recombinant polynucleotides are isolated from the host cells in which recombination occurs and are screened or selected in other host cells. For example, in some methods, it is advantageous to allow DNA repair to occur in a bacterial host strain, but to screen an expression product of recombinant polynucleotides in eucaryotic cells. The recombinant polynucleotides surviving screening or selection are sometimes useful products in themselves. In other instances, such recombinant polynucleotides are subjected to further recombination with each other or other substrates. Such recombination can be effected by the heteroduplex methods described above or any other shuffling methods. Further round(s) of recombination are followed by further rounds of screening or selection on an iterative basis. Optionally, the stringency of selection can be increased at each round.

The nature of screening or selection depends on the desired property sought to be acquired. Desirable properties of enzymes include high catalytic activity, capacity to confer resistance to drugs, high stability, the ability to accept a wider (or narrower) range of substrates, or the ability to function in nonnatural environments such as organic solvents. Other desirable properties of proteins include capacity to bind a selected target, secretion capacity, capacity to generate an immune response to a given target, lack of immunogenicity and toxicity to pathogenic microorganisms. Desirable properties of DNA or RNA polynucleotides sequences include capacity to specifically

bind a given protein target, and capacity to regulate expression of operably linked coding sequences. Some of the above properties, such as drug resistance, can be selected by plating cells on the drug. Other properties, such as the influence of a regulatory sequence on expression, can be screened by detecting appearance of the expression product of a reporter gene linked to the regulatory sequence. Other properties, such as capacity of an expressed protein to be secreted, can be screened by FACS™, using a labelled antibody to the protein. Other properties, such as immunogenicity or lack thereof, can be screened by isolating protein from individual cells or pools of cells, and analyzing the protein *in vitro* or in a laboratory animal.

VII. Variations

1. Demethylation

Most cell types methylate DNA in some manner, with the pattern of methylation differing between cells types. Sites of methylation include 5-methylcytosine (m^5C), N4-methylcytosine (m^4C) and N^6-methyladenine (m^6A), 5-hydroxymethylcytosine (hm^5C) and 5-hydroxymethyluracil (hm^5U). In *E. coli*, methylation is effected by *Dam* and *Dcm* enzymes. The methylase specified by the *dam* gene methylates the N6-position of the adenine residue in the sequence GATC, and the methylase specified by the *dcm* gene methylates the C5-position of the internal cytosine residue in the sequence CCWGG. DNA from plants and mammal is often subject to CG methylation meaning that CG or CNG sequences are methylated. Possible effects of methylated on cellular repair are discussed by references 18-20.

In some methods, DNA substrates for heteroduplex formation are at least partially demethylated on one or both strands, preferably the latter. Demethylation of substrate DNA promotes efficient and random repair of the heteroduplexes. In heteroduplexes formed with one strand dam-methylated and one strand unmethylated, repair is biased to the unmethylated strand, with the methylated strand serving as the template for correction. If neither strand is methylated, mismatch repair occurrs, but showes insignificant strand preference (23, 24).

Demethylation can be performed in a variety of ways. In some methods, substrate DNA is demethylated by PCR-amplification. In some instances, DNA demethylation is accomplished in one of the PCR steps in the heteroduplex formation

procedures described above. In other methods, an additional PCR step is performed to effect demethylation. In other methods, demethylation is effected by passaging substrate DNA through methylation deficient host cells (e.g. an *E. coli dam⁻dcm⁻* strain). In other methods, substrate DNA is demethylated *in vitro* using a demethylating enzymes. Demethylated DNA is used for heteroduplex formation using the same procedures described above. Heteroduplexes are subsequently introduced into DNA-repair-proficient but restriction-enzyme-defective cells to prevent degradation of the unmethylated heteroduplexes.

2. Sealing Nicks

Several of the methods for heteroduplex formation described above result in circular heteroduplexes bearing nicks in each strand. These nicks can be sealed before introducing heteroduplexes into host cells. Sealing can be effected by treatment with DNA ligase under standard ligating conditions. Ligation forms a phosphodiester bond to link two adjacent bases separated by a nick in one strand of double helix of DNA. Sealing of nicks increases the frequency of recombination after introduction of heteroduplexes into host cells.

3. Error Prone PCR Attendant To Amplification

Several of the formats described above include a PCR amplification step. Optionally, such a step can be performed under mutagenic conditions to induce additional diversity between substrates.

VIII. Other Shuffling Methods

The methods of heteroduplex formation described above can be used in conjunction with other shuffling methods. For example, one can perform one cycle of heteroduplex shuffling, screening or selection, followed by a cycle of shuffling by another method, followed by a further cycle of screening or selection. Other shuffling formats are described by WO 95/22625; US 5,605,793; US 5,811,238; WO 96/19256; Stemmer. *Science* 270, 1510 (1995); Stemmer et al., *Gene*, 164, 49-53 (1995); Stemmer. *Bio/Technology*, 13, 549-553 (1995); Stemmer. *Proc. Natl. Acad. Sci.* USA 91, 10747-10751 (1994); Stemmer. *Nature* 370, 389-391 (1994); Crameri et al., *Nature Medicine*, 2(1):1-3, (1996); Crameri et al., *Nature Biotechnology* 14, 315-319 (1996);WO 98/42727;

WO 98/41622; WO 98/05764 and WO 98/42728. WO 98/27230 (each of which is incorporated by reference in its entirety for all purposes).

IX. Protein Analogs

Proteins isolated by the methods also serve as lead compounds for the development of derivative compounds. The derivative compounds can include chemical modifications of amino acids or replace amino acids with chemical structures. The analogs should have a stabilized electronic configuration and molecular conformation that allows key functional groups to be presented in substantially the same way as a lead protein. In particular, the non-peptic compounds have spatial electronic properties which are comparable to the polypeptide binding region, but will typically be much smaller molecules than the polypeptides, frequently having a molecular weight below about 2 CHD and preferably below about 1 CHD. Identification of such non-peptic compounds can be performed through several standard methods such as self-consistent field (CSF) analysis, configuration interaction (CHI) analysis, and normal mode dynamics analysis. Computer programs for implementing these techniques are readily available. See Rein et al., *Computer-Assisted Modeling of Receptor-Ligand Interactions* (Alan Liss, New York, 1989).

IX. Pharmaceutical Compositions

Polynucleotides, their expression products, and secondary metabolites whose formation is catalyzed by expression products, generated by the above methods are optionally formulated as pharmaceutical compositions. Such a composition comprises one or more active agents, and a pharmaceutically acceptable carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, phosphate-buffered saline (PBS), 0.4% saline, 0.3% glycine, human albumin solution and the like. These solutions are sterile and generally free of particulate matter. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride and sodium is selected primarily based on fluid volumes, viscosities, and so forth, in accordance with the particular mode of administration selected.

EXAMPLES

EXAMPLE 1. Novel *Rhizobium Flaa* Genes From Recombination Of *Rhizobium Lupini Flaa* And *Rhizobium Meliloti FlaA*

Bacterial flagella have a helical filament, a proximal hook and a basal body with the flagellar motor (68). This basic design has been extensively examined in *E. coli* and *S. typhimurium* and is broadly applicable to many other bacteria as well as some archaea. The long helical filaments are polymers assembled from flagellin subunits, whose molecular weights range between 20,000 and 65,000, depending on the bacterial species (69). Two types of flagellar filaments, named plain and complex, have been distinguished by their electron microscopically determined surface structures (70). Plain filaments have a smooth surface with faint helical lines, whereas complex filaments exhibit a conspicuous helical pattern of alternating ridges and grooves. These characteristics of complex flagellar filaments are considered to be responsible for the brittle and (by implication) rigid structure that enables them to propel bacteria efficiently in viscous media (71-73). Whereas flagella with plain filaments can alternate between clockwise and counter clockwise rotation (68), all known flagella with complex filaments rotate only clockwise with intermittent stops (74). Since this latter navigation pattern is found throughout bacteria and archaea, it has been suggested that complex flagella may reflect the common background of an ancient, basic motility design (69).

Differing from plain bacterial flagella in the fine structure of their filaments dominated by conspicuous helical bands and in their fragility, the filaments are also resistant against heat decomposition (72). Schmitt et al. (75) showed that bacteriophage 7-7-1 specifically adsorbs to the complex flagella of *R.lupini* H 13-3 and requires motility for a productive infection of its host. Though the flagellins from *R. meliloti* and *R. lupini* are quite similar, bacteriophage 7-7-1 does not infect *R.meliloti*. Until now complex flagella have been observed in only three species of soil bacteria: *Pseudomonas rhodos* (73), *R.meliloti* (76), and *R.lupini* H13-3 (70, 72). Cells of *R.lupini* H13-3 posses 5 to 10 peritrichously inserted complex flagella, which were first isolated and analyzed by high resolution electron microscopy and by optical diffraction (70).

Maruyama et al. (77) further found that a higher content of hydrophobic amino acid residues in the complex filament may be one of the main reasons for the unusual properties of complex flagella. By measuring mass per unit length and obtaining three-dimensional reconstruction from electron micrographs, Trachtenberg et al. (73, 78)

suggested that the complex filaments of *R. lupini* are composed of functional dimers. Figure 6 shows the comparison between the deduced amino acid sequence of the *R. lupini* H13-3 FlaA and the deduced amino acid sequence of the *R. meliloti* FlaA. Perfect matches are indicated by vertical lines, and conservative exchanges are indicated by colons. The overall identity is 56%. The *R.lupini flaA* and *R.meliloti flaA* were subjected to *in vitro* heteroduplex formation followed by *in vivo* repair in order to create novel FlaA molecules and structures.

A. Methods

pRL20 containing *R. lupini* H13-3 *flaA* gene and pRM40 containing *R.meliloti flaA* gene are shown in Figs. 6A and 6B. These plasmids were isolated from *E. coli* SCS110 (free from dam- and dcm-type methylation).

About 3.0 pg. of unmethylated pRL20 and pRM40 DNA were digested with *Bam* HI and *Eco* RI, respectively, at 37°C for 1 hour. After agarose gel separation, the linearized DNA was purified with Wizard PCR Prep kit (Promega, WI, USA).

Equimolar concentrations (2.5 nM) of the linearized unmethylated pRL20 and pRM40 were mixed in 1 x SSPE buffer (180 mM NaCl, 1 mM EDTA, 10 mM NaH2PO4, pH 7.4). After heating at 96°C for 10 minutes, the reaction mixture was immediately cooled at 0°C for 5 minutes. The mixture was incubated at 68°C for 2 hour for heteroduplexes to form.

One microliter of the reaction mixture was used to transform 50 µl of *E. coli* ES 1301 *mutS*, *E. coli* SCS110 and *E. coli* JM109 competent cells. The transformation efficiency with *E. coli* JM109 competent cells was about seven times higher than that of *E. coli* SCS110 and ten times higher than that of *E. coli* ES1301 *mutS*, although the overall transformation efficiencies were 10-200 times lower than those of control transformations with the close, covalent and circular pUC19 plasmid.

Two clones were selected at random from the *E. coli* SCS110 transformants and two from *E. coli* ES1301 mutS transformants, and plasmid DNA was isolated from these four clones for further DNA sequencing analysis.

B. Results

Figure 7 shows (a) the sequence of SCS01 (clone#1 from *E. coli* SCS110 transformant library), (b) the sequence of SCS02 (clone #2 from *E. coli* SCS110

transformant library), (c) the sequence of ES01 (clone #1 from *E. coli* ES1301 transformant library), and (d) the sequence of ES02 (clone #2 from *E. coli* ES1301 transformant library). All four sequences were different from wild-type *R. lupini flaA* and *R. meliloti flaA* sequences. Clones SCS02, ES01 and ES02 all contain a complete open-reading frame, but SCS01 was truncated. Figure 8 shows that recombination mainly occurred in the loop regions (unmatched regions). The *flaA* mutant library generated from *R. meliloti flaA* and *R. lupini flaA* can be transformed into *E. coli* SCS110, ES1301, XL10-Gold and JM109, and transformants screened for functional FlaA recombinants.

EXAMPLE 2. Directed Evolution Of ECB Deacylase For Variants With Enhanced Specific Activity

Streptomyces are among the most important industrial microorganisms due to their ability to produce numerous important secondary metabolites (including many antibiotics) as well as large amounts of enzymes. The approach described here can be used with little modification for directed evolution of native *Streptomyces* enzymes, some or all of the genes in a metabolic pathways, as well as other heterologous enzymes expressed in *Streptomyces*.

New antifungal agents are critically needed by the large and growing numbers of immune-compromised AIDS, organ transplant and cancer chemotherapy patients who suffer opportunistic infections. Echinocandin B (ECB), a lipopeptide produced by some species of *Aspergillus*, has been studied extensively as a potential antifungal. Various antifungal agents with significantly reduced toxicity have been generated by replacing the linoleic acid side chain of *A. nidulans* echinocandin B with different aryl side chains (79-83). The cyclic hexapeptide ECB nucleus precursor for the chemical acylation is obtained by enzymatic hydrolysis of ECB using *Actinoplanes utahensis* ECB deacylase. To maximize the conversion of ECB into intact nucleus, this reaction is carried out at pH 5.5 with a small amount of miscible organic solvent to solubilize the ECB substrate. The product cyclic hexapeptide nucleus is unstable at pH above 5.5 during the long incubation required to fully deacylate ECB (84). The pH optimum of ECB deacylase, however, is 8.0-8.5 and its activity is reduced at pH 5.5 and in the presence of more than 2.5% ethanol (84). To improve production of ECB nucleus it is necessary to increase the activity of the ECB deacylase under these process-relevant conditions.

Relatively little is known about ECB deacylase. The enzyme is a heterodimer whose two subunits are derived by processing of a single precursor protein (83). The 19.9 kD α-subunit is separated from the 60.4 kD β-subunit by a 15-amino acid spacer peptide that is removed along with a signal peptide and another spacer peptide in the native organism. The polypeptide is also expressed and processed into functional enzyme in *Streptomyces lividans*, the organism used for large-scale conversion of ECB by recombinant ECB deacylase. The three-dimensional structure of the enzyme has not been determined, and its sequence shows so little similarity to other possibly related enzymes such as penicillin acylase that a structural model reliable enough to guide a rational effort to engineer the ECB deacylase will be difficult to build. We therefore decided to use directed evolution (85) to improve this important activity.

Protocols suitable for mutagenic PCR and random-priming recombination of the 2.4 kb ECB deacylase gene (73% G+C) have been described recently (86). Here, we further describe the use of heteroduplex recombination to generate new ECB deacylase with enhanced specific activity.

In this case, two *Actinoplanes utahensis* ECB deacylase mutants, M7-2 and M16, which show higher specific activity at pH 5.5 and in the presence of 10% MeOH were recombined using technique of the *in vitro* heteroduplex formation and *in vivo* mismatch repair .

Figure 12 shows the physical maps of plasmids pM7-2 and pM16 which contain the genes for the M7-2 and M16 ECB deacylase mutants. Mutant M7-2 was obtained through mutagenic PCR performed directly on whole *Streptomyces lividans* cells containing wild-type ECB deacylase gene, expressed from plasmid pSHP150-2*. *Streptomyces* with pM7-2 show 1.5 times the specific activity of cells expressing the wild-type ECB deacylase (86). Clone pM16 was obtained using the random-priming recombination technique as described (86, 87). It shows 2.4 times specific activity of the wild-type ECB deacylase clone.

A. Methods:

M7-2 and M16 plasmid DNA (pM7-2 and pM16) (Fig. 9) were purified from *E. coli* SCS110 (in separate reactions). About 5.0 μg of unmethylated M7-2 and M16 DNA were digested with *Xho* I and *Psh* AI, respectively, at 37°C for 1 hour (Fig.

10). After agarose gel separation, the linearized DNA was purified using a Wizard PCR Prep Kit (Promega, WI, USA).

Equimolar concentrations (2.0 nM) of the linearized unmethylated pM7-2 and pM16 DNA were mixed in 1 x SSPE buffer (1x SSPE: 180 mM NaCl, 1.0 mM EDTA, 10 mM NaH_2PO_4, pH 7.4). After heating at 96 °C for 10 minutes, the reaction mixture is immediately cooled at 0 °C for 5 minutes. The mixture was incubated at 68 °C for 3 hours to promote formation of heteroduplexes.

One microliter of the reaction mixture was used to transform 50 µl of *E.coli* ES1301 *mutS*, SCS110 and JM109 competent cells. All transformants from *E. coli* ES1301 *mutS* were pooled and *E. coli* SCS110 were pooled. A plasmid pool was isolated from each pooled library, and this pool was used to transform *S. lividans* TK23 protoplasts to form a mutant library for deacylase activity screening.

Transformants from the *S. lividans* TK23 libraries were screened for ECB deacylase activity with an *in situ* plate assay. Transformed protoplasts were allowed to regenerate on R2YE agar plates for 24 hr at 30°C and to develop in the presence of thiostrepton for 48 hours. When the colonies grew to the proper size, 6 ml of 0.7% agarose solution containing 0.5 mg/ml ECB in 0.1 M sodium acetate buffer (pH 5.5) was poured on top of each R2YE-agar plate and allowed to develop for 18-24 hr at 30°C. Colonies surrounded by a clearing zone larger than that of a control colony containing wild-type plasmid pSHP150-2*, were selected for further characterization.

Selected transformants were inoculated into 20 ml medium containing thiostrepton and grown aerobically at 30°C for 48 hours, at which point they were analyzed for ECB deacylase activity using HPLC. 100 µl of whole broth was used for a reaction at 30 °C for 30 minutes in 0.1 M NaAc buffer (pH 5.5) containing 10% (v/v) MeOH and 200 µg/ml of ECB substrate. The reactions were stopped by adding 2.5 volumes of methanol, and 20 µl of each sample were analyzed by HPLC on a 100 x 4.6 mm polyhydroxyethyl aspartamide column (PolyLC Inc., Columbia, MD, USA) at room temperature using a linear acetonitrile gradient starting with 50:50 of A:B (A = 93% acetonitrile, 0.1% phosphoric acid; B = 70% acetonitrile, 0.1% phosphoric acid) and ending with 30:70 of A:B in 22 min at a flow rate of 2.2 ml/min. The areas of the ECB and ECB nucleus peaks were calculated and subtracted from the areas of the

corresponding peaks from a sample culture of *S. lividans* containing pIJ702* in order to estimate the ECB deacylase activity.

2.0 ml pre-cultures of positive mutants were used to inoculate 50-ml medium and allowed to grow at 30°C for 96 hr. The supernatants were further concentrated to 1/30 their original volume using an Amicon filtration unit (Beverly, MA, USA) with molecular weight cutoff of 10 kD. The resulting enzyme samples were diluted with an equal volume of 50 mM KH_2PO4 (pH 6.0) buffer and were applied to Hi-Trap ion exchange column (Pharmacia Biotech, Piscataway, NJ, USA) . The binding buffer was 50 mM KH_2PO_4 (pH 6.0), and the elution buffer was 50 mM KH_2PO_4 (pH 6.0) containing 1.0 M NaCl. A linear gradient from 0 to 1.0 M NaCl was applied in 8 column volumes with a flow rate of 2.7 ml/min. The ECB deacylase fraction eluting at 0.3 M NaCl was concentrated and the buffer was exchanged for 50 mM KH_2PO_4 (pH 6.0) using Centricon-10 units. Enzyme purity was verified by SDS-PAGE using Coomassie Blue stain, and the concentration was determined using the Bio-Rad Protein Assay Reagent (Hercules, CA, USA).

A modified HPLC assay was used to determine the activities of the ECB deacylase mutants on ECB substrate (84). Four μg of each purified ECB deacylase mutant was used for activity assay reaction at 30°C for 30 minutes in 0.1 M NaAc buffer (pH 5.5) containing 10% (v/v) MeOH and different concentrations of ECB substrate. Assays were performed in duplicate. The reactions were stopped by adding 2.5 volumes of methanol. and the HPLC assays were carried out as described above. The absorbance values were recorded. and the initial rates were calculated by least-squares regression of the time progress curves from which the Km and the kcat were calculated.

Activities as a function of pH were measured for the purified ECB deacylases at 30°C at different pH values: 5, 5.5 and 6 (0.1 M acetate buffer); 7, 7.5, 8 and 8.5 (0.1 M phosphate buffer); 9 and 10 (0.1 M carbonate buffer) using the HPLC assay. Stabilities of purified ECB deacylases were were determined at 30°C in 0.1 M NaAc buffer (pH 5.5) containing 10% methanol. Samples were withdrawn at different time intervals. and the residual activity was measured in the same buffer with the HPLC assay described above.

B. Results

Fig. 11 shows that after one round of applying this heteroduplex repair technique on the mutant M7-2 and M16 genes. one mutant (M15) from about 500 original transformants was found to possess 3.1 times the specific activity of wild-type.

Wild type and evolved M15 ECB deacylases were purified and their kinetic parameters for deacylation of ECB were determined by HPLC. The evolved deacylases M15 has an increased catalytic rate constant, k_{cat} by 205%. The catalytic efficiency (k_{cat}/K_m) of M20 is enhanced by a factor of 2.9 over the wild-type enzyme.

Initial rates of deacylation with the wild type and M15 at different pH values from 5 to 10 were determined at 200 μg/ml of ECB. The recombined M15 is more active than wild type at pH 5-8. Although the pH dependence of the enzyme activity in this assay is not strong, there is a definite shift of 1.0-1.5 units in the optimum to lower pH, as compared to wild type.

The time courses of deactivation of the purified ECB deacylase mutant M15 was measured in 0.1 M NaAc (pH 5.5) at 30°C. No significant difference in stability was observed between wild type and mutant M15.

The DNA mutations with respect to the wild type ECB deacylase sequence and the positions of the amino acid substitutions in the evolved variants M7-2, M16 and M15 are summarized in Figure 12.

The heteroduplex recombination technique can recombine parent sequences to create novel progeny. Recombination of the M7-2 and M16 genes yielded M15, whose activity is higher than any of its parents (Fid. 13). Of the six base substitutions in M15, five (at positions α50, α171, β57, β129 and β340) were inherited from M7-2, and the other one (β30) came from M16.

This approach provides an alternative to existing methods of DNA recombination and is particularly useful in recombining large genes or entire operons. This method can be used to create recombinant proteins to improve their properties or to study structure-function relationship.

EXAMPLE 3. Novel Thermostable *Bacillus Subtilis* Subtilisin E Variants

This example demonstrates the use *in vitro* heteroduplex formation followed by *in vivo* repair for combining sequence information from two different sequences in order to improve the thermostability of *Bacillus subtilis* subtilisin E.

Genes RC1 and RC2 encode thermostable *B. sublilis* subtilisin E variants (88). The mutations at base positions 1107 in RC1 and 995 in RC2 (Figure 14), giving rise to amino acid substitutions Asn218/Ser (N218S) and Asn181/Asp (N181 ID), lead to improvements in subtilisin E thermostability; the remaining mutations, both synonymous and nonsynonymous, have no detectable effects on thermostability. At 65°C, the single variants N181D and N218S have approximately 3-fold and 2-fold longer half-lives, respectively, than wild subtilisin E. and variants containing both mutations have half-lives that are 8-fold longer (88). The different half-lives in a population of subtilisin E variants can therefore be used to estimate the efficiency by which sequence information is combined. In particular. recombination between these two mutations (in the absence of point mutations affecting thermostability) should generate a library in which 25% of the population exhibits the thermos/ability of the double mutant. Similarly, 25% of the population should exhibit wild-type like stability, as N181D and N218S are eliminated at equal frequency. We used the fractions of the recombined population as a diagnostic

A. Methods

The strategy underlying this example is shown in Fig. 15.

Subtilisin E thermostable mutant genes RC1 and RC2 (Fig. 14) are 986-bp fragments including 45 nt of subtilisin E prosequence, the entire mature sequence and 113 nt after the stop codon. The genes were cloned between *Bam* HI and *Nde* I in *E. coli/B. subtilis* shuttle vector pBE3. resulting in pBE3-1 and pBE3-2, respectively. Plasmid DNA pBE3-1 and pBE3-2 was isolated from *E.coli* SCS110.

About 5.0 µg of ummethylated pBE3-1 and pBE3-2 DNA were digested with *Bam* HI and *Nde* I. respectively. at 37°C for 1 hour. After agarose gel separation. equimolar concentrations (2.0nM) of the linearized unmethylated pBE3-1 and pBE3-2 were mixed in 1 x SSPE buffer (180 mM NaCl. 1.0 mM EDTA. 10 mM NaH_2PO_4. pH 7.4). After heating at 96°C for 10 minutes. the reaction mixture was immediately cooled at 0°C for 5 min. The mixture was incubated at 68°C for 2 hr for heteroduplexes to form.

One microliter of the reaction mixture was used to transform 50 µl of *E. coli* ES 1301 *mutS*. *E. coli* SCS110 and *E. coli* HB101 competent cells.

The transformation efficiency with *E. coli* HB101 competent cells was about ten times higher than that of *E.coli* SCS110 and 15 times higher than that of *E. coli* ES1301 *mutS*. But in all these cases, the transformation efficiencies were 10-250 times lower than that of the transformation with closed, covalent and circular control pUC19 plasmids.

Five clones from *E. coli* SCS110 mutant library and five from *E. coli* ES1301 *mutS* library were randomly chosen, and plasmid DNA was isolated using a QIAprep spin plasmid miniprep kit for further DNA sequencing analysis.

About 2.000 random clones from *E. coli* HB101 mutant library were pooled and total plasmid DNA was isolated using a QIAGEN-100 column. 0.5-4.0 ug of the isolated plasmid was used to transform *Bacillus subtilis* DB428 as described previously (88).

About 400 transformants from the *Bacillus subtilis* DB428 library were subjected to screening. Screening was performed using the assay described previously (88), on succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. *B. subtilis* DB428 containing the plasmid library were grown on LB plates containing kanamycin (20 µg/ml) plates. After 18 hours at 37°C single colonies were picked into 96-well plates containing 200 µl SG/kanamycin medium per well. These plates were incubated with shaking at 37°C for 24 hours to let the cells to grow to saturation. The cells were spun down, and the supernatants were sampled for the thermostability assay.

Two replicates of 96-well assay plates were prepared for each growth plate by transferring 10 µl of supernatant into the replica plates. The subtilisin activities were then measured by adding 100 µl of activity assay solution (0.2 mM succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0, 37°C). Reaction velocities were measured at 405 nm to over 1.0 min in a ThermoMax microplate reader (Molecular Devices, Sunnyvale CA). Activity measured at room temperature was used to calculate the fraction of active clones (clones with activity less than 10% of that of wild type were scored as inactive). Initial activity (A_i) was measured after incubating one assay plate at 65°C for 10 minutes by immediately adding 100 µl of prewarmed (37°C) assay solution (0.2 mM succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, 100 mM Tris-HCl, pH 8.0, 10 mM

$CaCl_2$, pH 8.0) into each well. Residual activity (Ar) was measured after 40 minute incubation.

B. Results

In vitro heteroduplex formation and *in vivo* repair was carried out as described above. Five clones from *E. coli* SCS110 mutant library and five from *E. coli* ES1301 *mutS* libraries were selected at random and sequenced. Fig. 14 shows that four out of the ten clones were different from the parent genes. The frequency of occurrence of a particular point mutation from parent RC1 or RC2 in the resulting genes ranged from 0% to 50%, and the ten point mutations in the heteroduplex have been repaired without strong strand-specific preference.

Since none of the ten mutations locates within the dcm site, the mismatch repair appears generally done via the *E. coli* long-patch mismatch repair systems. The system repairs different mismatches in a strand-specific manner using the state of N6-methylation of adenine in GATC sequences as the major mechanism for determining the strand to be repaired. With heteroduplexes methylated at GATC sequences on only one DNA strand, repair was shown to be highly biased to the unmethylated strand, with the methylated strand serving as the template for correction. If neither strand was methylated, mismatch repair occurred, but showed little strand preference (23, 24). These results shows that it is preferable to demethylate the DNA to be recombined to promote efficient and random repair of the heteroduplexes.

The rates of subtilisin E thermo-inactivation at 65°C were estimated by analyzing the 400 random clones from the *Bacillus subtilis* DB428 library. The thermostabilities obtained from one 96-well plate are shown in Figure 16, plotted in descending order. About 12.9% of the clones exhibited thermostability comparable to the mutant with the N181D and N218S double mutations. Since this rate is only half of that expected for random recombination of these two markers, it indicates that the two mismatches at positions 995 and 1107 within the heteroduplexes have been repaired with lower position randomness.

Sequence analysis of the clone exhibiting the highest thermostability among the screened 400 transformants from the *E. coli* SCS110 heteroduplex library confirmed the presence of both N181D and N218S mutations. Among the 400 transformants from the *B.sublilis* DB428 library that were screened, approximately 91%

of the clones expressed N181D- and/or N218S-type enzyme stabilities, while about 8.0% of the transformants showed only wild-type subtilisin E stability.

Less than 1.0% inactive clone was found, indicating that few new point mutations were introduced in the recombination process. This is consistent with the fact that no new point mutations were identified in the ten sequenced genes (Figure 14). While point mutations may provide useful diversity for some *in vitro* evolution applications, they can also be problematic for recombination of beneficial mutations, especially when the mutation rate is high.

EXAMPLE 4. Optimizing Conditions For The Heteroduplex Recombination.

We have found that the efficiency of heteroduplex recombination can differ considerably from gene to gene [17,57]. In this example, we investigate and optimize a variety of parameters that improve recombination efficiency.
DNA substrates used in this example were site-directed mutants of green fluorescent protein from *Aequorea victoria*. The GFP mutants had a stop codon(s) introduced at different locations along the sequence that abolished their fluorescence. Fluorescent wild type protein could be only restored by recombination between two or more mutations. Fraction of fluorescent colonies was used as a measure of recombination efficiency.

A. Methods

About 2-4 μg of each parent plasmid was used in one recombination experiment. One parent plasmid was digested with *Pst* I endonuclease another parent with *Eco*RI. Linearized plasmids were mixed together and 20 x SSPE buffer was added to the final concentration 1x (180 mM NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.4). The reaction mixture was heated at 96°C for 4 minutes, immediately transferred on ice for 4 minutes and the incubation was continued for 2 hours at 68°C.

Target genes were amplified in a PCR reaction with primers corresponding to the vector sequence of pGFP plasmid. Forward primer: 5'-CCGACTGGAAAGCGGGCAGTG-3', reverse primer 5'-CGGGGCTGGCTTAACTATGCGG-3'. PCR products were mixed together and purified using Qiagen PCR purification kit. Purified products were mixed with 20 x SSPE buffer and hybridized as described above. Annealed products were precipitated with ethanol or

purified on Qiagen columns and digested with *Eco*RI and *Pst*I enzymes. Digested products were ligated into *Pst*I and *Eco*RI digested pGFP vector.

dUTP was added into PCR reaction at final concentrations 200 μM, 40μM, 8 μM, 1.6 μM, 0.32 μM. PCR reaction and subsequent cloning procedures were performed as described above.

Recombinant plasmids were transformed into XL10 *E. coli* strain by a modified chemical transformation method. Cells were plated on ampicillin containing LB agar plates and grown overnight at 37°C, followed by incubation at room temperature or at 4°C until fluorescence developed.

B. Results.

1. Effect of ligation on recombination efficiency.

Two experiments have been performed to test the effect of breaks in the DNA heteroduplex on the efficiency of recombination. In one experiment heteroduplex plasmid was treated with DNA ligase to close all existing single-strand breaks and was transformed in identical conditions as an unligated sample (see Table 1). The ligated samples show up to 7-fold improvement in recombination efficiency over unligated samples.

In another experiment, dUTP was added into PCR reaction to introduce additional breaks into DNA upon repair by uracyl N-glycosylase in the host cells. Table 2 shows that dUMP incorporation significantly suppressed recombination, the extent of suppression increasing with increased dUTP concentration.

2. Effect of plasmid size on the efficiency of heteroduplex formation.

Plasmid size was a significant factor affecting recombination efficiency. Two plasmids pGFP (3.3 kb) and a *Bacillus* shuttle vector pCT1 (about 9 kb) were used in preparing circular heteroduplex-like plasmids following traditional heteroduplex protocol. For the purpose of this experiment (to study the effect of plasmid size on duplex formation), both parents had the same sequences. While pGFP formed about 30-40% of circular plasmid, the shuttle vector yielded less than 10% of this form.

Increase in plasmid size decreases concentration of the ends in the vicinity of each and makes annealing of very long (>0.8 kb) ends that are single-stranded more difficult. This difficulty is avoided by the procedure shown in Fig. 3, in which heteroduplex formation occurs between substrates in vector-free form, and, heteroduplexes are subsequently inserted into a vector.

3. Efficiency of Recombination vs. Distance Between Mutations

A series of GFP variants was recombined pairwise to study the effect of distance between mutations on the efficiency of recombination. Parental genes were amplified by PCR, annealed and ligated back into pGFP vector. Heteroduplexes were transformed into XL10 *E.coli* strain.

The first three columns in Table 3 show the results of three independent experiments and demonstrate the dependence of recombination efficiency on the distance between mutations. As expected recombination becomes less and less efficient for very close mutations.

However, it is still remarkable that long-patch repair has been able to recombine mutations separated by only 27 bp.

The last line in Table 3 represents recombination between one single and one double mutants. Wild type GFP could only be restored in the event of double crossover with each individual crossover occurring in the distance of 99 bp only, demonstrating the ability of this method to recombine multiple, closely-spaced mutations.

4. Elimination Of The Parental Double Strands From Heteroduplex Preparations.

Annealing of substrates in vector-free form offers size-advantages relative to annealing of substrates as components of vectors, but does not allow selection for heteroduplexes relative to homoduplexes simply by transformation into host. Asymmetric PCR reactions with only one primer for each parent seeded with appropriate amount of previously amplified and purified gene fragment were run for 100 cycles, ensuring a 100-fold excess of one strand over another. Products of these asymmetrical reactions were mixed and annealed together producing only a minor amount of nonrecombinant duplexes. The last column in Table 3 shows the recombination efficiency obtained from these enriched heteroduplexes. Comparison of the first three

columns with the fourth one demonstrates the improvement achieved by asymmetric synthesis of the parental strands.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

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WHAT IS CLAIMED IS:

1. A method for evolving a polynucleotide toward acquisition of a desired property, comprising

(a) incubating a population of parental polynucleotide variants under conditions to generate annealed polynucleotides comprising heteroduplexes;

(b) exposing the heteroduplexes to a cellular DNA repair system to convert the heteroduplexes to parental polynucleotide variants or recombined polynucleotide variants;

(c) screening or selecting the recombined polynucleotide variants for the desired property.

2. The method of claim 1, wherein the heteroduplexes are exposed to the cellular DNA repair system in vitro.

3. The method of claim 2, wherein the cellular DNA repair system comprises cellular extracts.

4. The method of claim 1, further comprising introducing the heteroduplexes into cells, whereby the heteroduplexes are exposed to the DNA repair system of the cells in vivo.

5. The method of claim 4, wherein the annealed polynucleotides further comprise homoduplexes and the introducing step selects for transformed cells comprising the heteroduplexes relative to transformed cells comprising homoduplexes.

6. The method of claim 4, wherein a first polynucleotide variant is provided as a component of a first vector, and a second polynucleotide variant is provided as a component of a second vector, and the method further comprises converting the first and second vectors to linearized forms in which the first and second polynucleotide variants occur at opposite ends, whereby in the incubating step single-stranded forms of the first linearized vector reanneal with each other to form linear first vector, single-stranded forms of the second linearized vector reanneal with each other to form linear second vector, and single-stranded linearized forms of the first and second vectors anneal with each to form a circular heteroduplex bearing a nick in each strand, and the

introducing step selects for transformed cells comprising the circular heteroduplexes relative to the linear first and second vector.

7. The method of claim 6, wherein the first and second vectors are converted to linearized forms by PCR.

8. The method of claim 6, wherein the first and second vectors are converted to linearized forms by digestion with first and second restriction enzymes.

9. The method of claim 1, wherein the population of polynucleotide variants are provided in double stranded form, and the method further comprising converting the double stranded polynucleotides to single stranded polynucleotides before the annealing step.

10. The method of claim 1, wherein the converting step comprises:

conducting asymmetric amplification of the first and second double stranded polynucleotide variants to amplify a first strand of the first polynucleotide variant, and a second strand of the second polynucleotide variant, whereby the first and second strands anneal in the incubating step to form a heteroduplex.

11. The method of claim 10, wherein the first and second double-stranded polyncueltoides variants are provided in vector-free form, and the method further comprises incorporating the heteroduplex into a vector.

12. The method of claim 4 wherein the population of polynucleotides comprises first and second polynucleotides provided in double stranded form, and the method further comprises incorporating the first and second polynucleotides as components of first and second vectors, whereby the first and second polynucleotides occupy opposite ends of the first and second vectors, whereby in the incubating step single-stranded forms of the first linearized vector reanneal with each other to form linear first vector, single-stranded forms of the second linearized vector reanneal with each other to form linear second vector, and single-stranded linearized forms of the first and second vectors anneal with each to form a circular heteroduplex bearing a nick in each

strand, and the introducing step selects for transformed cells comprises the circular heteroduplexes relative to the linear first and second vector.

13. The method of claim 4, further comprising sealing nicks in the heteroduplexes to form covalently-closed circular heteroduplexes before the introducing step.

14. The method of claim 11, wherein the first and second polynucleotides are obained from chromosomal DNA..

15. The method of claim 1, further comprising repeating steps (a)-(c) whereby the incubating step in a subsequent cycle is performed on recombinant variants from a previous cycle.

16. The method of claim 1, wherein the polynucleotide variants encode a polypeptide.

17. The method of claim 1, wherein the population of polynucleotide variants comprises at least 20 variants.

18. The method of claim 1, wherein the population of polynucleotide variants are at least 10 kb in length.

19. The method of claim 1, wherein the population of polynucleotide variants comprises natural variants.

20. The method of claim 1, wherein the population of polynucleotides comprises variants generated by mutagenic PCR.

21. The method of claim 1, wherein the population of polynucleotide variants comrises variants generated by site directed mutagenesis.

22. The method of claim 1, wherein the cells are bacterial cells.

23. The method of claim 1, further comprising at least partially demethylating the population of variant polynucleotides.

24. The method of claim 23, whether the at least partially demethylating step is performed by PCR amplification of the population of variant polynucleotides.

25. The method of claim 23, wherein the at least partially demethylating step is performed by amplification of the population of variant polynucelotides in host cells.

26. The method of claim 25, wherein the host cells are defective in a gene encoding a methylase enzyme.

27. The method of claim 1, wherein the population of variant polynucleotide variants comprises at least 5 polynucleotides having at least 90% sequence identity with one another.

28. The method of claim 1, further comprising isolating a screened recombinant variant.

29. The method of claim 28, further comprising expressing a screened recombinant variant to produce a recombinant protein.

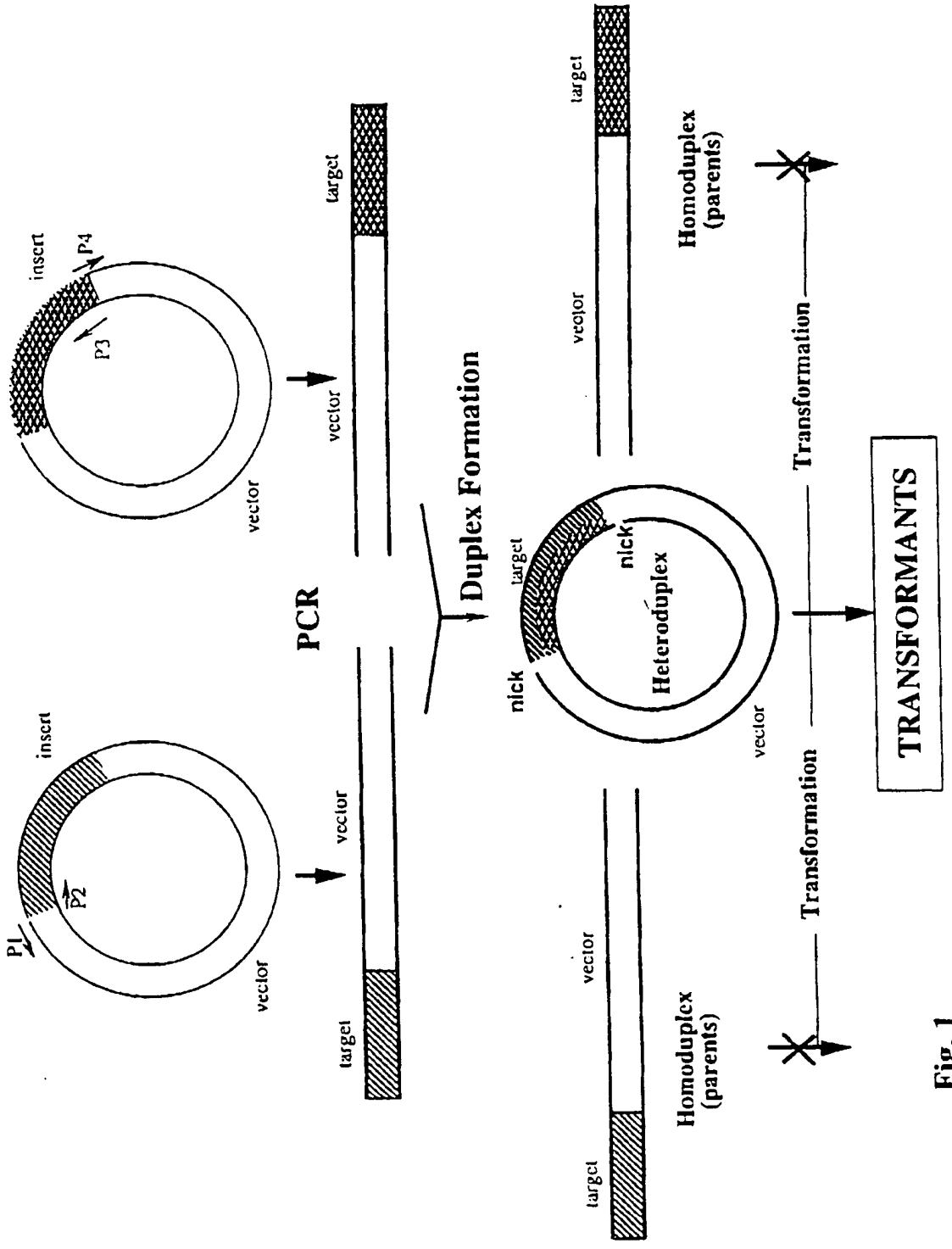
30. The method of claim 29 further comprising formulating the recombinant protein with a carrier to form a pharmaceutical composition.

31. The method of claim 1, wherein the polynucleotide variants encode enzymes selected from the group consisting of proteases, lipases, amylases, cutinases, cellulases, amylases, oxidases, peroxidases and phytases.

32. The method of claim 1, wherein the polynucleotide variants encode a polypeptide selected from the group consisting of insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erthropoietin, luteinizing hormone, chorionic gonadotropin, hyperthalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon, thrombopoietin (TPO), and prolactin.

33. The method of claim 1, wherein the polynucleotide variants encode a plurality of enzymes forming a metabolic pathway.

34. The method of claim 1, wherein the polynucleotide variants are in concatemeric form.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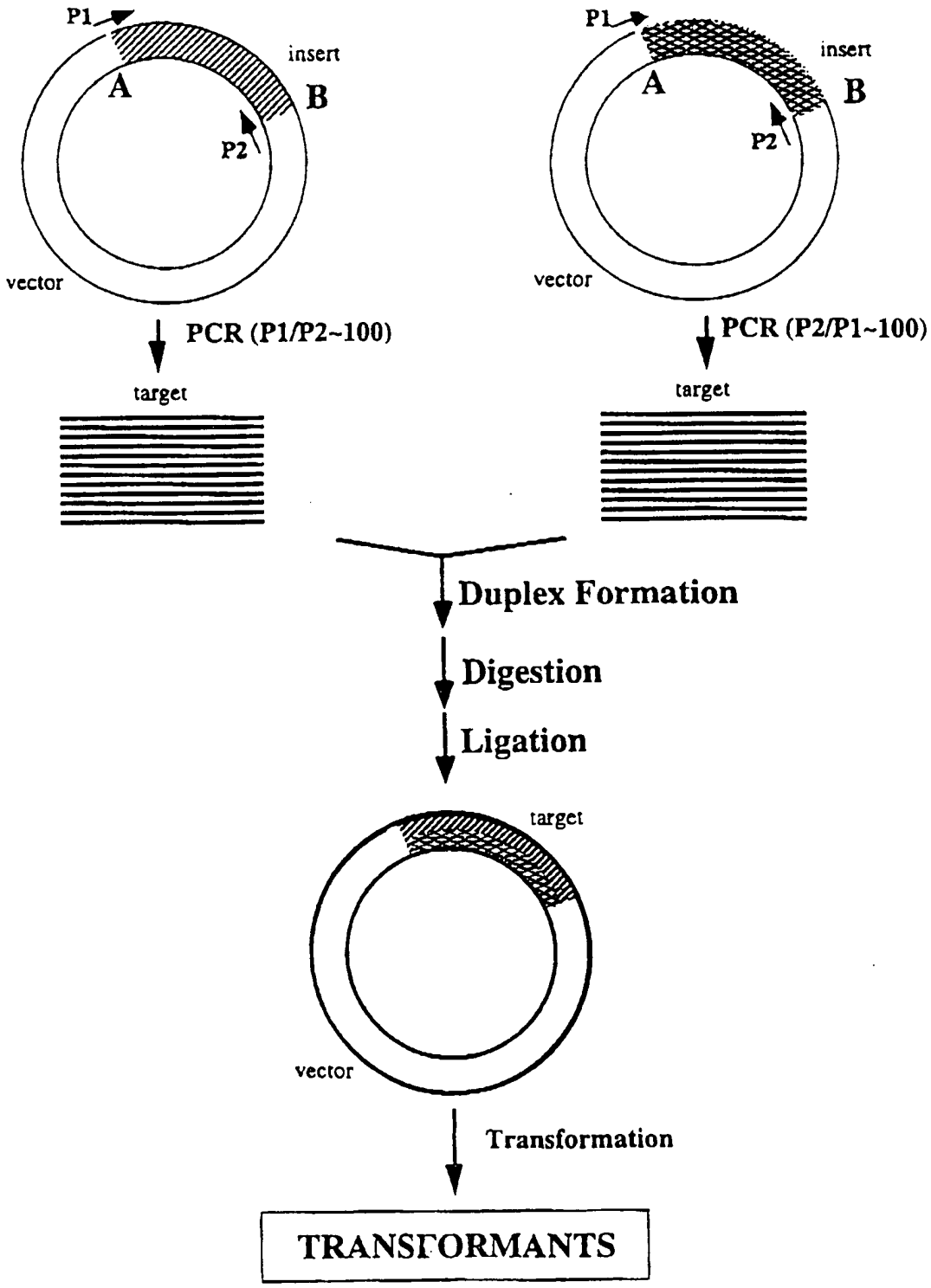
P4
P3
vector
target
vector
PCR
Duplex Formation
target
vector
Homoduplex
(parents)
P1
P2
insert
vector
target
vector
nick
target
nick
Heteroduplex
vector
Transformation
Transformation
TRANSFORMANTS
target
vector
Homoduplex
(parents)

Fig. 1

A
insert
vector
Enzyme A
vector
target
Enzyme B
vector
vector
Duplex Formation
target
nick
nick
Heteroduplex
vector
vector
target
Homoduplex
(parents)
Transformation
Transformation
TRANSFORMANTS

Fig. 2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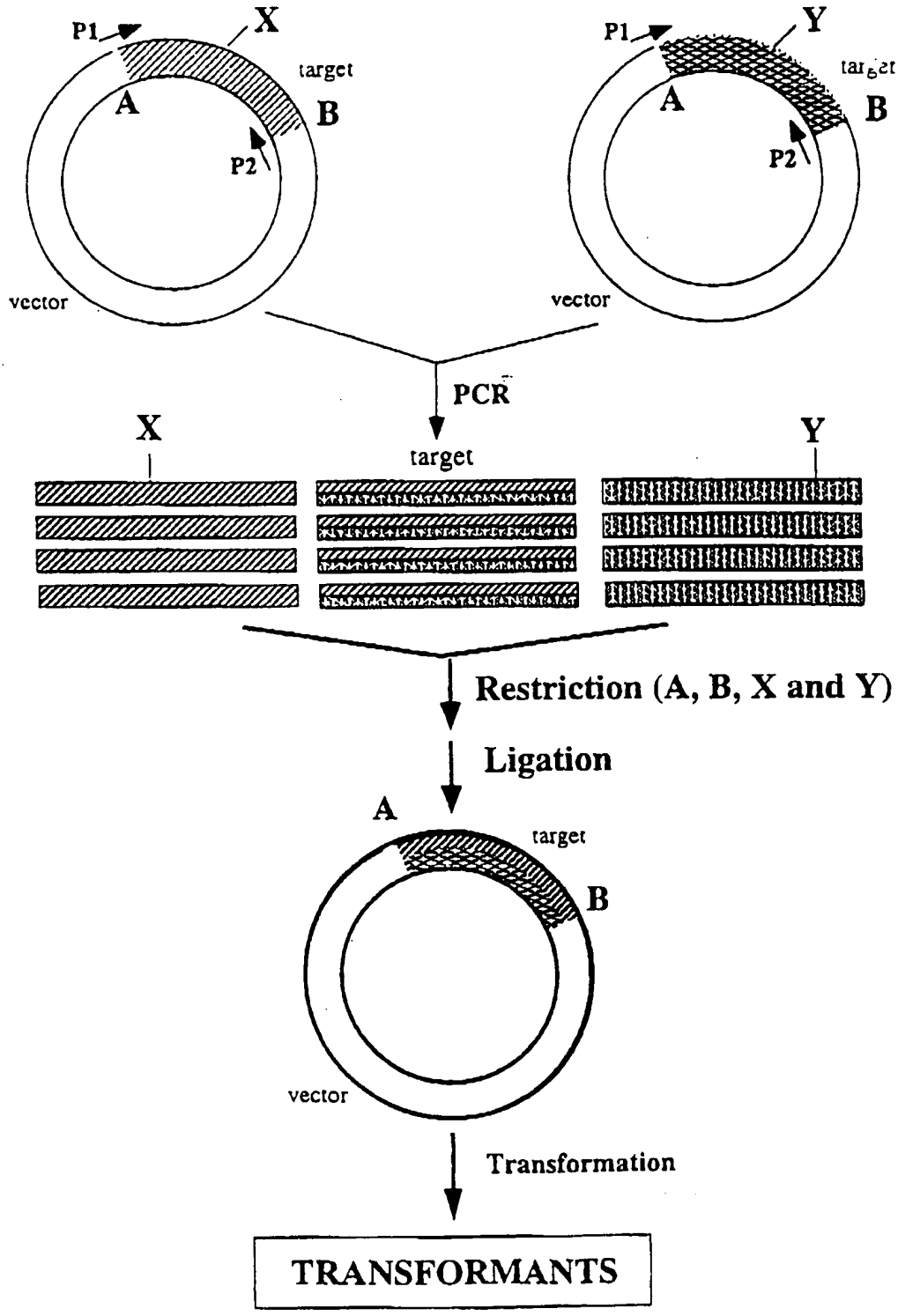
A
B
P2
vector
PCR (P1/P2~100)
target
P1
insert
A
B
P2
vector
PCR (P2/P1~100)
target
Duplex Formation
Digestion
Ligation
target
vector
Transformation
TRANSFORMANTS

Fig. 3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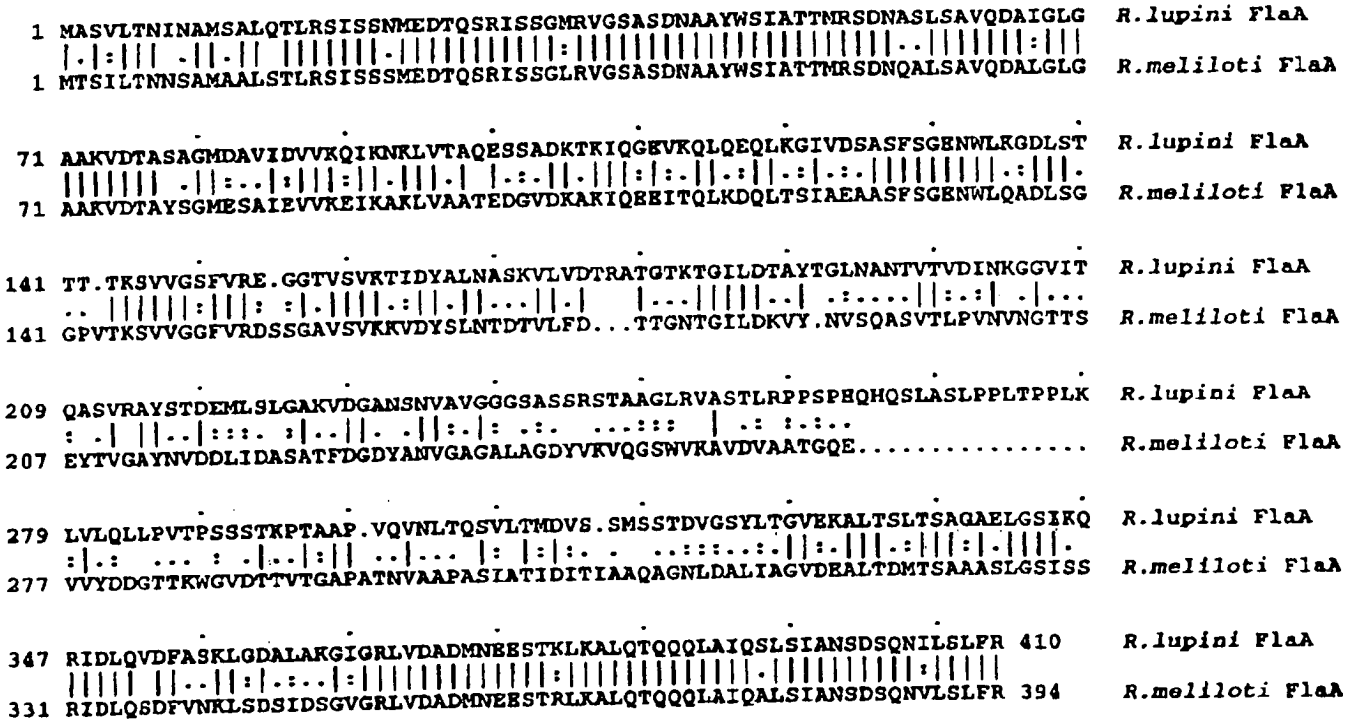
X
target
A
B
P2
vector
P1
Y
target
A
B
P2
vector
PCR
X
target
Y
Restriction (A, B, X and Y)
Ligation
A
target
B
vector
Transformation
TRANSFORMANTS

Fig. 4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71 AAKVDTASAGMDAVIDVVKQIKNKLVTAQESSADKTKIQGEVKQLQEQLKGIVDSASFSGENWLKGDLST R.lupini FlaA
71 AAKVDTAYSGMESAIEVVKEIKAKLVAATEDGVDKAKIQEEITQLKDQLTSIAEAASFSGENWLQADLSG R.meliloti FlaA
141 TT.TKSVVGSFVRE.GGTVSVKTIDYALNASKVLVDTRATGTKTGILDTAYTGLNANTVTVDINKGGVIT R.lupini FlaA
141 GPVTKSVVGGFVRDSSGAVSVKKVDYSLNTDTVLFD...TTGNTGILDKVY.NVSQASVTLPVNVNGTTS R.meliloti FlaA
209 QASVRAYSTDEMLSLGAKVDGANSNVAVGGGSASSRSTAAGLRVASTLRPPSPHQHQSLASLPPLTPPLK R.lupini FlaA
207 EYTVGAYNVDDLIDASATFDGDYANVGAGALAGDYVKVQGSWVKAVDVAATGQE..... R.meliloti FlaA
279 LVLQLLPVTPSSSTKPTAAP.VQVNLTQSVLTMDVS.SMSSTDVGSYLTGVEKALTSLTSAQAELGSIKQ R.lupini FlaA
277 VVYDDGTTKWGVDTTVTGAPATNVAAPASIATIDITIAAQAGNLDALIAGVDEALTDMTSAAASLGSISS R.meliloti FlaA
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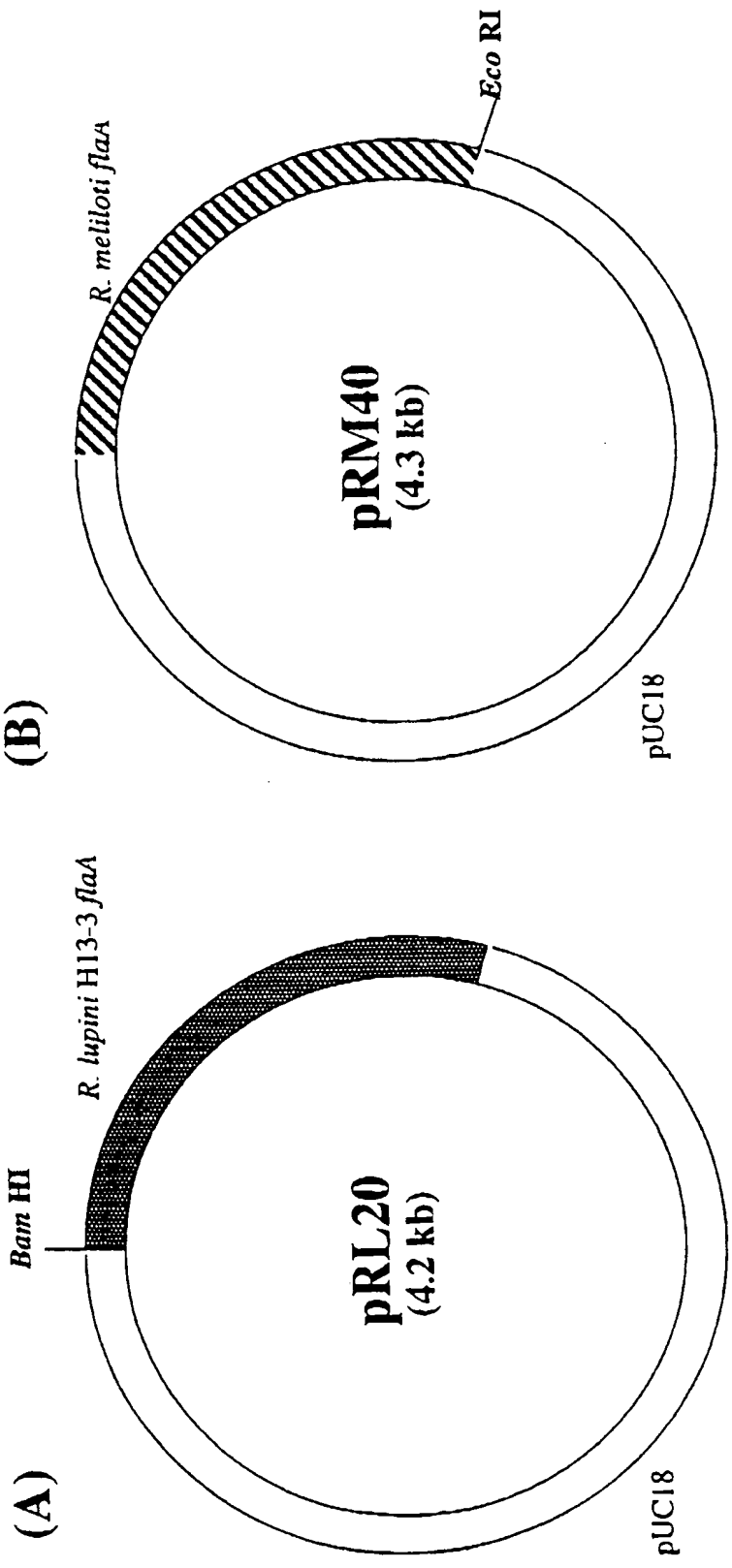
Fig. 5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(4.2 kb)
pUC18
(B)
R. meliloti flaA
Eco RI
pRM40
(4.3 kb)
pUC18

Fig. 6A & 6B

(a)

```
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SCS01   CCAGGGCAACTATGCTCTTCAGGGCGGTAACAGCTACGTCAAGGTCGAAAACGTCTGGGT..........TCGA.GCTGAG.........ACCGCATCA
SCS01   ACACCAGTCGCTGGCAAGTTTGCCGCCGCTTACACCGCCGCTGAAGCTGGTACTGCAGCTGCTGCCGGTGACgCCATCATCGTCGACGAAACCAACAGCG
SCS01   GCGCCGGTGCAGGTAAACCTCACCCAGTCGgTCCTGACCATGGATGTCAGCTCGATGAGCTCGACGGATGTCGGCAGCTACCTCACGGGCGTGGAAAAGG
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SCS01   ATCGCAAACAGCGACTCGCAGAACATTCTGTCGCTGTTCCGTTAA
```

(b)

```
SCS02   ATGACGAGCATTCTCACCAACAACTCCGCAATGGCCGCGCTTTCCGGAGTGCGCTCGATCTCTTCCAGCATGGAAGACACGCAGAGCCGCATCTCCTCCG
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SCS02   ATCGCCAACTCGGACTCGCAGAACGTCCTGTCGCTCTTCCGCTAA
```

Fig. 7A & 7B

(c)

```
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ES01    CTCTCACCAGCCTGACGAGCGCTGGCGCTGAACTCGGCTCCATCTCCTCGCGCATCGACCTGCAGAGCGAATTCGTCAACAAGCTCTCGGACTCGATCGA
ES01    GTCGGGCGTCGGCCGTCTCGTCGACGCGGACATGAACGAGGAGTCGACCCGCCTCAAGGCCCTGCAGACCCAGCAGCAGCTCGCCATCCAGGCCCTGTCG
ES01    ATCGCCAACTCGGACTCGCAGAACGTCCTGTCGCTCTTCCGCTAA
```

(d)

```
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Fig. 7C & 7D

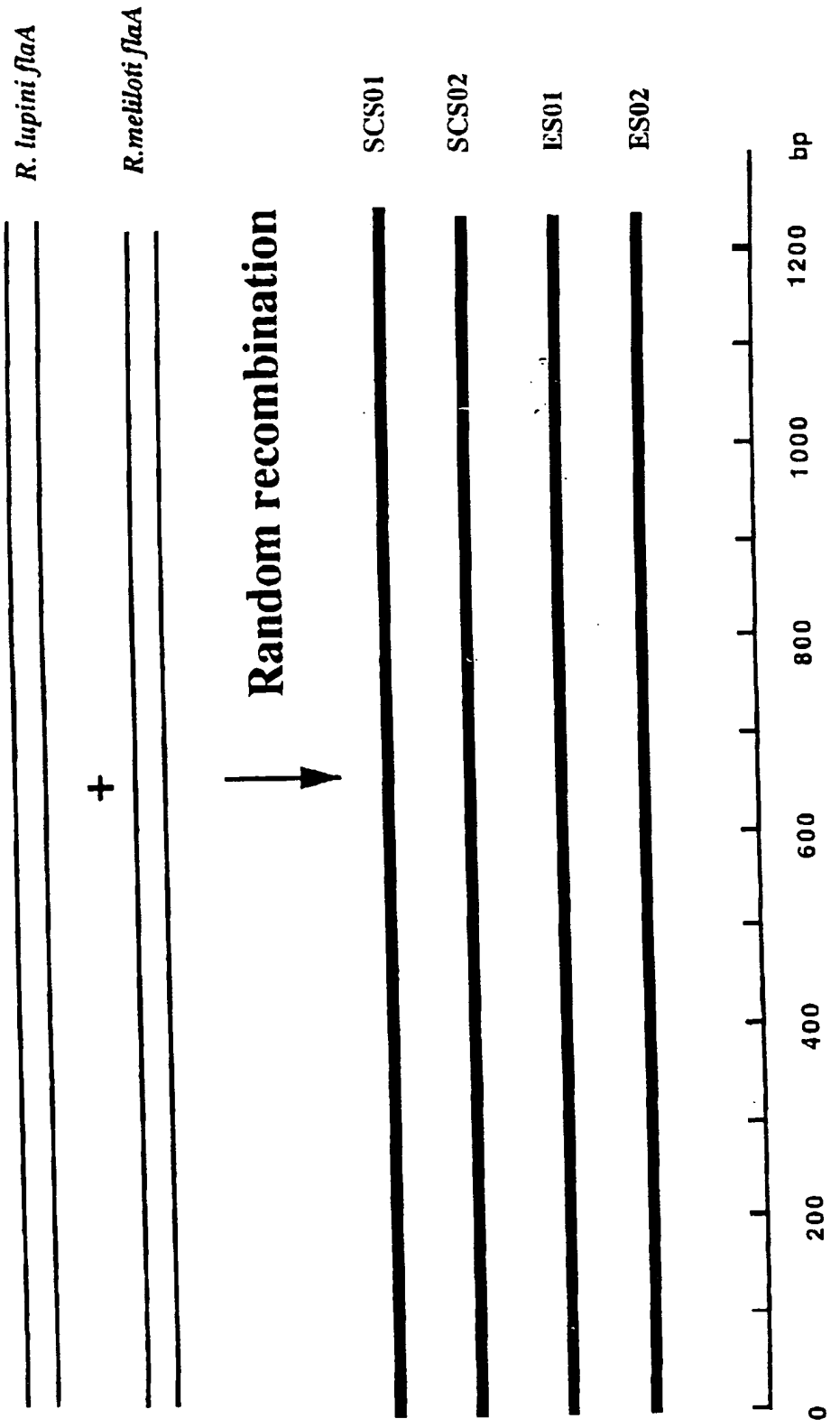
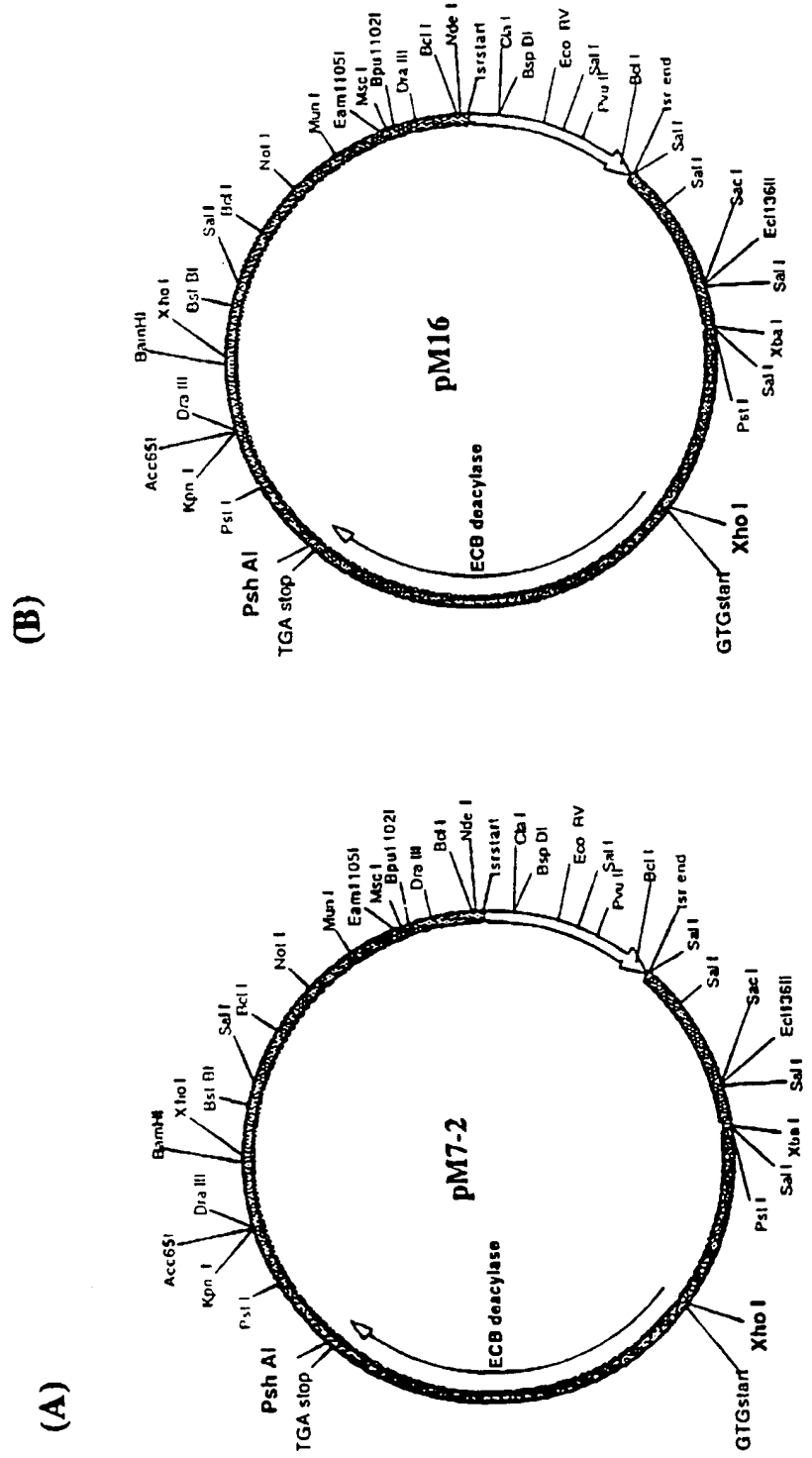

R. lupini flaA
R.meliloti flaA
+
Random recombination
SCS01
SCS02
ES01
ES02
0
200
400
600
800
1000
1200
bp

Fig. 8


(B)
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ECB deacylase
Psh AI
TGA stop
GTGstart
Xho I
Acc65I
Kpn I
Dra III
BamHI
Xho I
Bst BI
Sal I
Bcl I
Not I
Mun I
Eam1105I
Msc I
Bpu1102I
Dra III
Bcl I
Nde I
1srstart
Cla I
Bsp DI
Eco RV
Sal I
Pvu II
Bcl I
1sr end
Sal I
Sal I
Sac I
Ecl136II
Sal I
Xba I
Sal I
Pst I
(A)
pM7-2
ECB deacylase
Psh AI
TGA stop
GTGstart
Xho I
Acc65I
Kpn I
Dra III
BamHI
Xho I
Bst BI
Sal I
Bcl I
Not I
Mun I
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Msc I
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Bcl I
Nde I
1srstart
Cla I
Bsp DI
Eco RV
Sal I
Pvu II
Bcl I
1sr end
Sal I
Sal I
Sac I
Ecl136II
Sal I
Xba I
Sal I
Pst I

Fig. 9

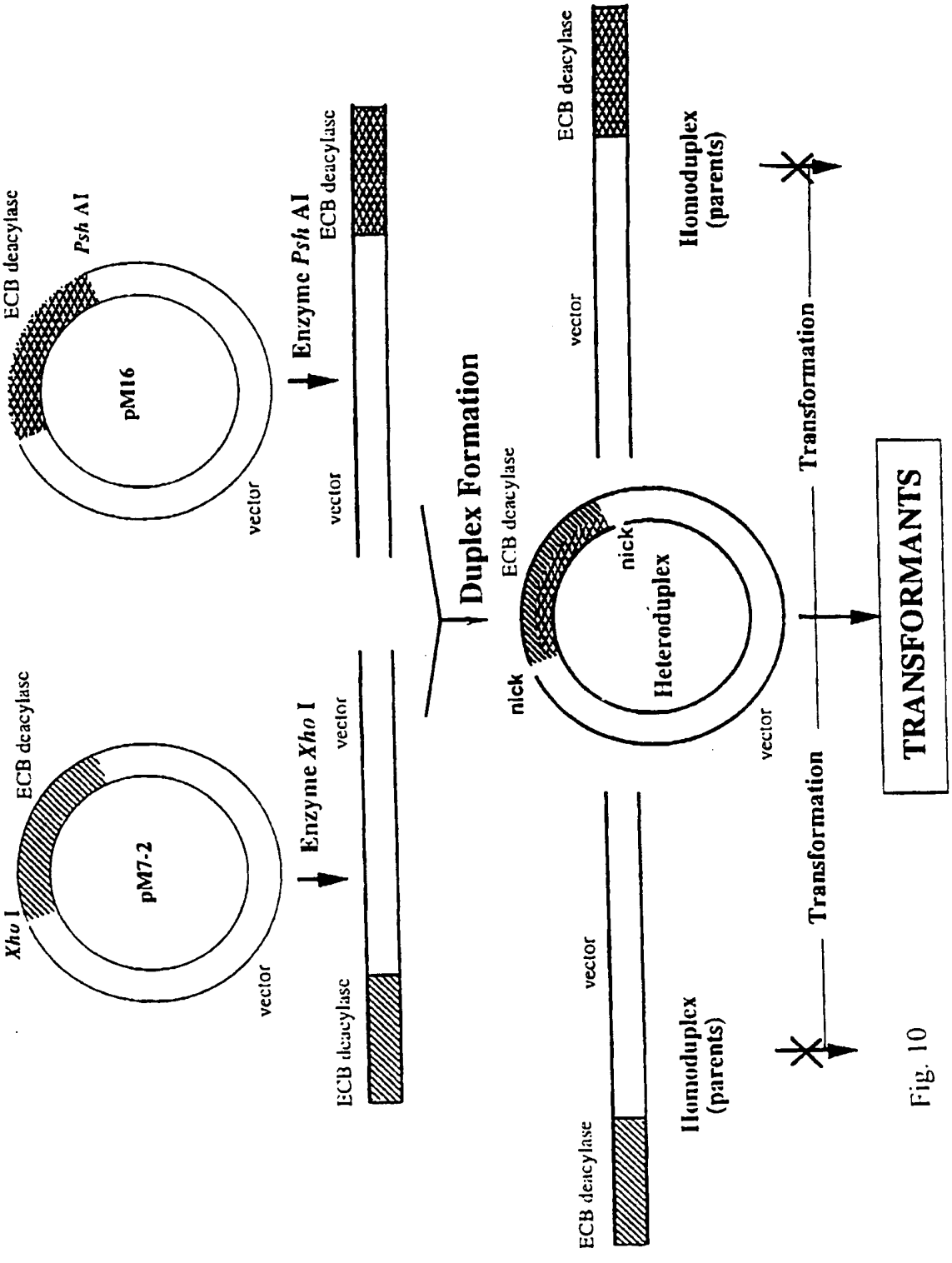
ECB deacylase
Psh AI
pM16
vector
Enzyme Psh AI
ECB deacylase
vector
Xho I
ECB deacylase
pM7-2
vector
Enzyme Xho I
vector
ECB deacylase
Duplex Formation
ECB deacylase
vector
Homoduplex
(parents)
nick
nick
Heteroduplex
ECB deacylase
vector
Transformation
Transformation
ECB deacylase
vector
Homoduplex
(parents)
TRANSFORMANTS

Fig. 10

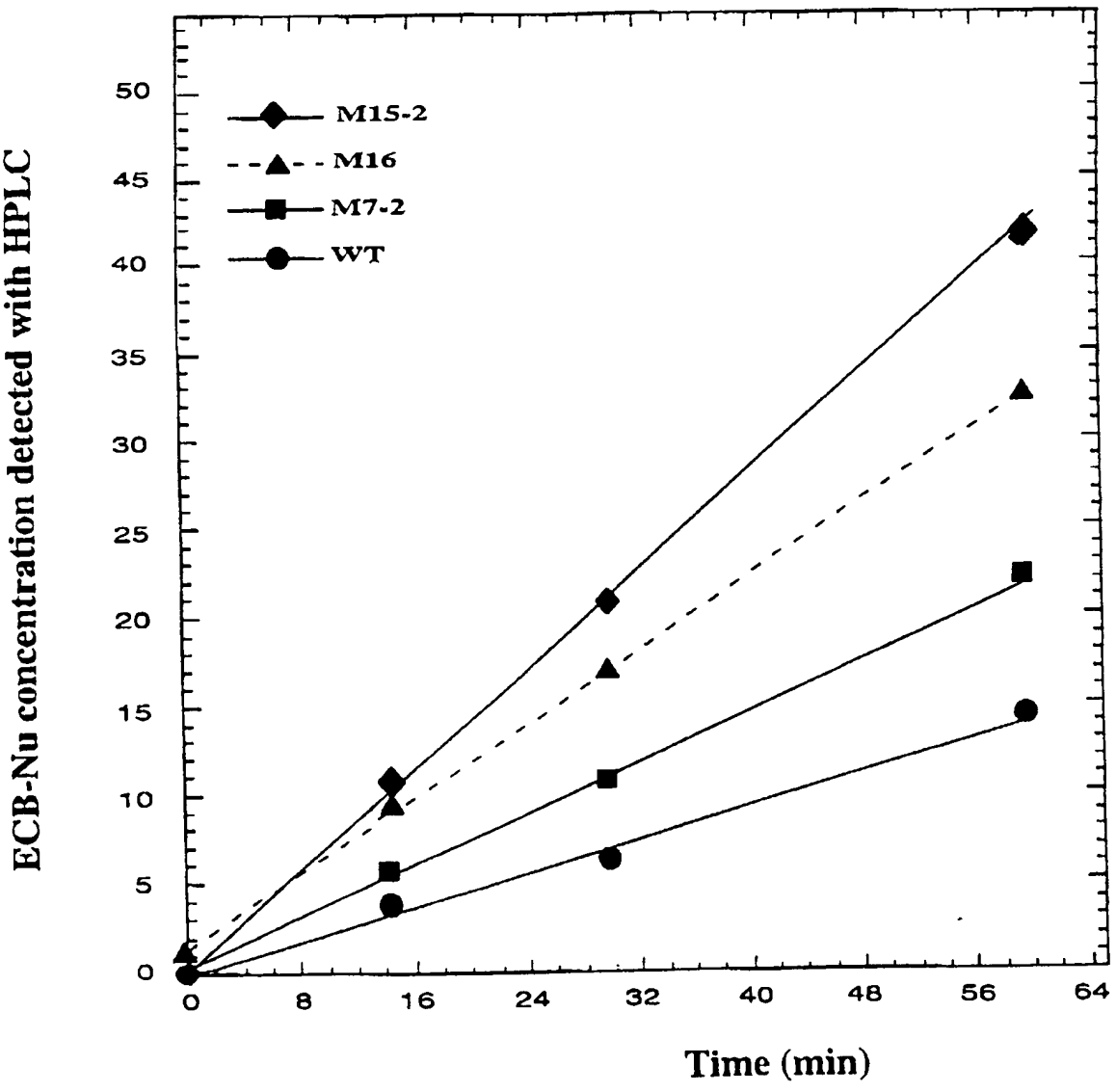
ECB-Nu concentration detected with HPLC
Time (min)
M15-2
M16
M7-2
WT
0
5
10
15
20
25
30
35
40
45
50
0
8
16
24
32
40
48
56
64

Fig. 11

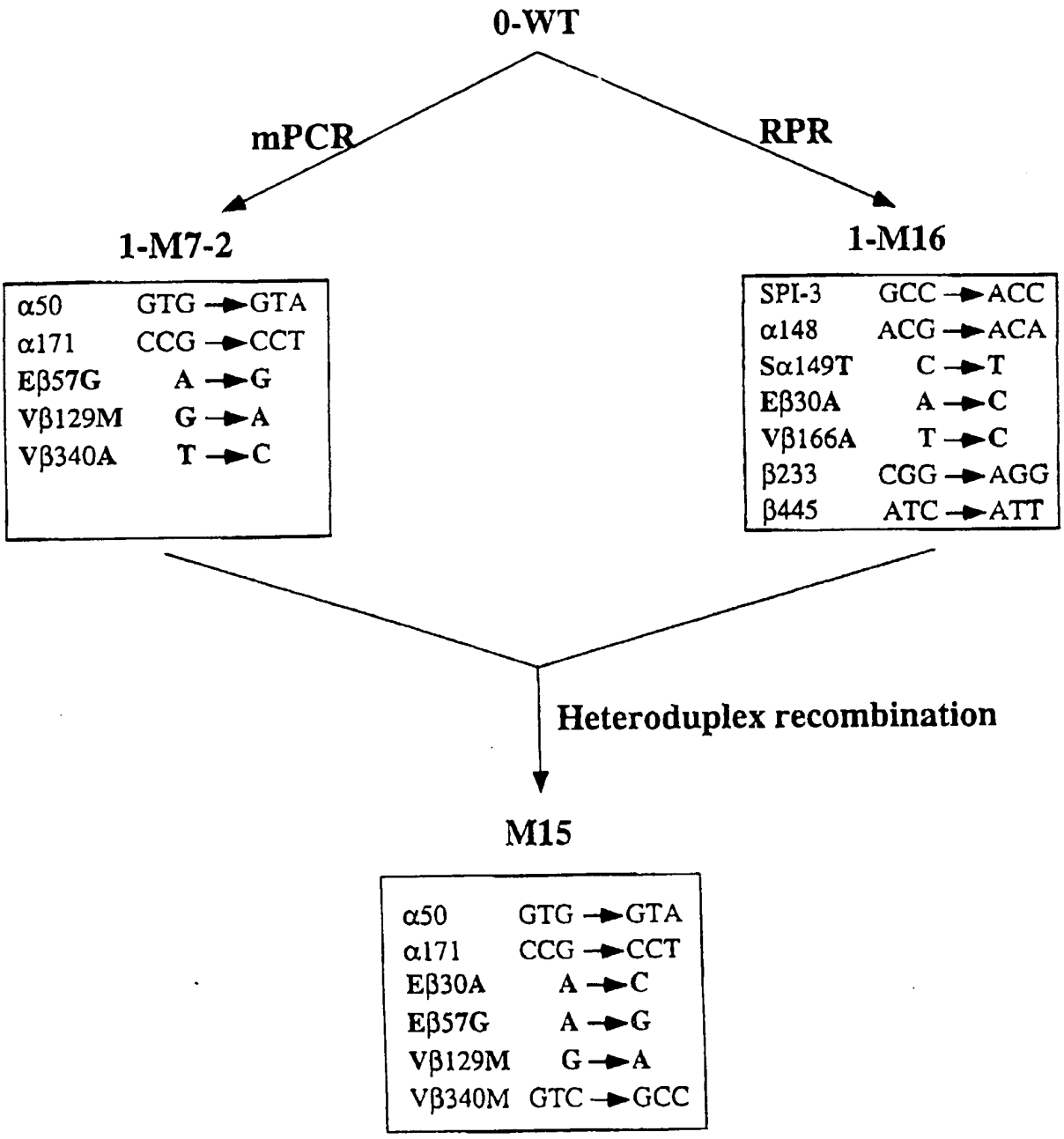
0-WT
mPCR
RPR
1-M7-2
α50 GTG → GTA
α171 CCG → CCT
Eβ57G A → G
Vβ129M G → A
Vβ340A T → C
1-M16
SPI-3 GCC → ACC
α148 ACG → ACA
Sα149T C → T
Eβ30A A → C
Vβ166A T → C
β233 CGG → AGG
β445 ATC → ATT
Heteroduplex recombination
M15
α50 GTG → GTA
α171 CCG → CCT
Eβ30A A → C
Eβ57G A → G
Vβ129M G → A
Vβ340M GTC → GCC

Fig. 12

```
1     CTGCAGCGTGCCCAGCTGTTCGTGGTGGTGATCGCGGCCGCGCTGGCCGCCGTCGCGGTC
61    GCCGCCGCCGGGCCGATCGAGTTCGTCGCCTTCGTCGTGCCGCAGATCGCCCTGCGGCTC
121   TGCGGCGGCAGCCGGCCGCCCCTGCTCGCCTCGGCGATGCTCGGCGCGCTGCTGGTGGTC
181   GGCGCCGACCTGGTCGCTCAGATCGTGGTGGCGCCGAAGGAGCTGCCGGTCGGCCTGCTC
241   ACCGCGATGATCGGCACCCCGTACCTGCTCTGGCTCCTGCTTCGGCGATCAAGAAAGGTG
301   AGCGGATGAACGCCCGCCTGCGTGGCGAGGGCCTGCACCTCGCGTACGGGGACCTGACCG
361   TGATCGACGGCCTCGACGTCGACGTGCACGACGGGCTGGTCACCACCATCATCGGGCCCA
421   ACGGGTGCGGCAAGTCGACGCTGCTCAAGGCGCTCGGCCGGCTGCTGCGCCCGACCGGCG
481   GGCAGGTGCTGCTGGACGGCCGCCGCATCGACCGGACCCCCACCCGTGACGTGGCCCGGG
541   TGCTCGGCGTGCTGCCGCAGTCGCCCACCGCGCCCGAAGGGCTCACCGTCGCCGACCTGG
601   TGATGCGCGGCCGGCACCCGCACCAGACCTGGTTCCGGCAGTGGTCGCGCGACGACGAGG
661   ACCAGGTCGCCGACGCGCTGCGCTGGACCGACATGCTGGCGTACGCGGACCGCCCGGTGG
721   ACGCCCTCTCCGGCGGTCAGCGCCAGCGCGCCTGGATCAGCATGGCGCTGGCCCAGGGCA
781   CCGACCTGCTGCTGCTGGACGAGCCGACCACCTTCCTCGACCTGGCCCACCAGATCGACG
841   TGCTGGACCTGGTCCGCCGGCTGCACGCCGAGATGGGCCGGACCGTGGTGATGGTGCTGC
901   ACGACCTGAGCCTGGCCGCCCGGTACGCCGACCGGCTGATCGCGATGAAGGACGGCCGGA
961   TCGTGGCGAGCGGGGCGCCGGACGAGGTGCTCACCCCGGCGCTGCTGGAGTCGGTCTTCG
1021  GGCTGCGCGCGATGGTGGTGCCCGACCCGGCGACCGGCACCCCGCTGGTGATCCCCCTGC
1081  CGCGCCCCGCCACCTCGGTGCGGGCCTGAAATCGATGAGCGTGGTTGCTTCATCGGCCTG
1141  CCGAGCGATGAGAGTATGTGGGCGGTAGAGCGAGTCTCGAGGGGGAGATGCCGCCGTGAC
                                                          V   T

1201  GTCCTCGTACATGCGCCTGAAAGCAGCAGCGATCGCCTTCGGTGTGATCGTGGCGACCGC
  5     S   S   Y   M   R   L   K   A   A   A   I   A   F   G   V   I   V   A   T   A

1261  AGCCGTGCCGTCACCCGCTTCCGGCAGGGAACATGACGGCGGCTATGCGGCCCTGATCCG
 23     A   V   P   S   P   A   S   G   R   E   H   D   G   G   Y   A   A   L   I   R

1321  CCGGGCCTCGTACGGCGTCCCGCACATCACCGCCGACGACTTCGGGAGCCTCGGTTTCGG
 43     R   A   S   Y   G   V   P   H   I   T   A   D   D   F   G   S   L   G   F   G

1381  CGTCGGGTACGTGCAGGCCGAGGACAACATCTGCGTCATCGCCGAGAGCGTAGTGACGGC
 63     V   G   Y   V   Q   A   E   D   N   I   C   V   I   A   E   S   V   V   T   A

1441  CAACGGTGAGCGGTCGCGGTGGTTCGGTGCGACCGGGCCGGACGACGCCGATGTGCGCAG
```

Fig. 13A

```
 83   N  G  E  R  S  R  W  F  G  A  T  G  P  D  D  A  D  V  R  S

1501 CGACCTCTTCCACCGCAAGGCGATCGACGACCGCGTCGCCGAGCGGCTCCTCGAAGGGCC
103   D  L  F  H  R  K  A  I  D  D  R  V  A  E  R  L  L  E  G  P

1561 CCGCGACGGCGTGCGGGCGCCGTCGGACGACGTCCGGGACCAGATGCGCGGCTTCGTCGC
123   R  D  G  V  R  A  P  S  D  D  V  R  D  Q  M  R  G  F  V  A

1621 CGGCTACAACCACTTCCTACGCCGCACCGGCGTGCACCGCCTGACCGACCCGGCGTGCCG
143   G  Y  N  H  F  L  R  R  T  G  V  H  R  L  T  D  P  A  C  R

1681 CGGCAAGGCCTGGGTGCGCCCGCTCTCCGAGATCGATCTCTGGCGTACGTCGTGGGACAG
163   G  K  A  W  V  R  P  L  S  E  I  D  L  W  R  T  S  W  D  S

1741 CATGGTCCGGGCCGGTTCCGGGGCGCTGCTCGACGGCATCGTCGCCGCGACGCCACCTAC
183   M  V  R  A  G  S  G  A  L  L  D  G  I  V  A  A  T  P  P  T

1801 AGCCGCCGGGCCCGCGTCAGCCCCGGAGGCACCCGACGCCGCCGCGATCGCCGCCGCCCT
203   A  A  G  P  A  S  A  P  E  A  P  D  A  A  A  I  A  A  A  L

1861 CGACGGGACGAGCGCGGGCATCGGCAGCAACGCGTACGGCCTCGGCGCGCAGGCCACCGT
223   D  G  T  S  A  G  I  G  S  N  A  Y  G  L  G  A  Q  A  T  V

1921 GAACGGCAGCGGGATGGTGCTGGCCAACCCGCACTTCCCGTGGCAGGGCGCCGCACGCTT
243   N  G  S  G  M  V  L  A  N  P  H  F  P  W  Q  G  A  A  R  F

1981 CTACCGGATGCACCTCAAGGTGCCCGGCCGCTACGACGTCGAGGGCGCGGCGCTGATCGG
263   Y  R  M  H  L  K  V  P  G  R  Y  D  V  E  G  A  A  L  I  G

2041 CGACCCGATCATCGGGATCGGGCACAACCGCACGGTCGCCTGGAGCCACACCGTCTCCAC
283   D  P  I  I  G  I  G  H  N  R  T  V  A  W  S  H  T  V  S  T

2101 CGCCCGCCGGTTCGTGTGGCACCGCCTGAGCCTCGTGCCCGGCGACCCCACCTCCTATTA
303   A  R  R  F  V  W  H  R  L  S  L  V  P  G  D  P  T  S  Y  Y
```

Fig. 13B

2161 CGTCGACGGCCGGCCCGAGCGGATGCGCGCCCGCACGGTCACGGTCCAGACCGGCAGCGG
323 V D G R P E R M R A R T V T V Q T G S G

2221 CCCGGTCAGCCGCACCTTCCACGACACCCGCTACGGCCCGGTGGCCGTGATGCCGGGCAC
343 P V S R T F H D T R Y G P V A V M P G T

2281 CTTCGACTGGACGCCGGCCACCGCGTACGCCATCACCGACGTCAACGCGGGCAACAACCG
363 F D W T P A T A Y A I T D V N A G N N R

2341 CGCCTTCGACGGGTGGCTGCGGATGGGCCAGGCCAAGGACGTCCGGGCGCTCAAGGCGGT
383 A F D G W L R M G Q A K D V R A L K A V

2401 CCTCGACCGGCACCAGTTCCTGCCCTGGGTCAACGTGATCGCCGCCGACGCGCGGGGCGA
403 L D R H Q F L P W V N V I A A D A R G E

2461 GGCCCTCTACGGCGATCATTCGGTCGTCCCCCGGGTGACCGGCGCGCTCGCTGCCGCCTG
423 A L Y G D H S V V P R V T G A L A A A C

2521 CATCCCGGCGCCGTTCCAGCCGCTCTACGCCTCCAGCGGCCAGGCGGTCCTGGACGGTTC
443 I P A P F Q P L Y A S S G Q A V L D G S

2581 CCGGTCGGACTGCGCGCTCGGCGCCGACCCCGACGCCGCGGTCCCGGGCATTCTCGGCCC
463 R S D C A L G A D P D A A V P G I L G P

2641 GGCGAGCCTGCCGGTGCGGTTCCGCGACGACTACGTCACCAACTCCAACGACAGTCACTG
483 A S L P V R F R D D Y V T N S N D S H W

2701 GCTGGCCAGCCCGGCCGCCCCGCTGGAAGGCTTCCCGCGGATCCTCGGCAACGAACGCAC
503 L A S P A A P L E G F P R I L G N E R T

2761 CCCGCGCAGCCTGCGCACCCGGCTCGGGCTGGACCAGATCCAGCAGCGCCTCGCCGGCAC
523 P R S L R T R L G L D Q I Q Q R L A G T

2821 GGACGGTCTGCCCCGGCAAGGGCTTCACCACCGCCCGGCTCTGGCAGGTCATGTTCGGCAA
543 D G L P G K G F T T A R L W Q V M F G N

Fig. 13C

```
2881 CCGGATGCACGGCGCCGAACTCGCCCGCGACGACCTGGTCGCGCTCTGCCGCCGCCAGCC
563   R  M  H  G  A  E  L  A  R  D  D  L  V  A  L  C  R  R  Q  P

2941 GACCGCGACCGCCTCGAACGGCGCGATCGTCGACCTCACCGCGGCCTGCACGGCGCTGTC
583   T  A  T  A  S  N  G  A  I  V  D  L  T  A  A  C  T  A  L  S

3001 CCGCTTCGATGAGCGTGCCGACCTGGACAGCCGGGGCGCGCACCTGTTCACCGAGTTCGC
603   R  F  D  E  R  A  D  L  D  S  R  G  A  H  L  F  T  E  F  A

3061 CCTCGCGGGCGGAATCAGGTTCGCCGACACCTTCGAGGTGACCGATCCGGTACGCACCCC
623   L  A  G  G  I  R  F  A  D  T  F  E  V  T  D  P  V  R  T  P

3121 GCGCCGTCTGAACACCACGGATCCGCGGGTACGGACGGCGCTCGCCGACGCCGTGCAACG
643   R  R  L  N  T  T  D  P  R  V  R  T  A  L  A  D  A  V  Q  R

3181 GCTCGCCGGCATCCCCCTCGACGCGAAGCTGGGAGACATCCACACCGACAGCCGCGGCGA
663   L  A  G  I  P  L  D  A  K  L  G  D  I  H  T  D  S  R  G  E

3241 ACGGCGCATCCCCATCCACGGTGGCCGCGGGGAAGCAGGCACCTTCAACGTGATCACCAA
683   R  R  I  P  I  H  G  G  R  G  E  A  G  T  F  N  V  I  T  N

3301 CCCGCTCGTGCCGGGCGTGGGATACCCGCAGGTCGTCCACGGAACATCGTTCGTGATGGC
703   P  L  V  P  G  V  G  Y  P  Q  V  V  H  G  T  S  F  V  M  A

3361 CGTCGAACTCGGCCCGCACGGCCCGTCGGGACGGCAGATCCTCACCTATGCGCAGTCGAC
723   V  E  L  G  P  H  G  P  S  G  R  Q  I  L  T  Y  A  Q  S  T

3421 GAACCCGAACTCACCCTGGTACGCCGACCAGACCGTGCTCTACTCGCGGAAGGGCTGGGA
743   N  P  N  S  P  W  Y  A  D  Q  T  V  L  Y  S  R  K  G  W  D

3481 CACCATCAAGTACACCGAGGCGCAGATCGCGGCCGACCCGAACCTGCGCGTCTACCGGGT
763   T  I  K  Y  T  E  A  Q  I  A  A  D  P  N  L  R  V  Y  R  V

3541 GGCACAGCGGGGACGCTGACCCACGTCACGCCGGCTCGGCCCGTGCGGGGGCGCAGGGCG
```

Fig. 13D

783 A Q R G R

3601 CCGATCGTCTCTGCATCGCCGGTCAGCCGGGGCCTGCGTCGACCGGCGGCGGCCGGTCGA
3661 CGCCCGCGTCCCGGCGCAGCGACTGGCTGAAGCGCCAGGCGTCGGCGGCCCGGGGCAGGT
3721 TGTTGAACATCACGTACGCCGGGCCGCCGTCGAGGATGCCGGCGAGGTGTGCCAGCTCGG
3781 CATCCGTGTACACATGCCGGGCGCCGGTGATGCCGTGCAGCCGGTAATAGGCCATCGGCG
3841 TCAGACTGCGGCGCAGGAACGGGTCGGCGGCGTGGGTCAGGTCCAGCTCCTGGCACAAGC
3901 CCTCGACCACCTCGTCCGGCCACGGGCCGCGCGGCTCCCACAACAGCCGGACACCGGCCG
3961 GCCGGCGCGCTCGGGCGCAGAACTCACGCAGTCGCGCGATGGCGGGTTCGGTCGGCCGGA
4021 AACTCGCCGGGCACTGCAG

Fig. 13E

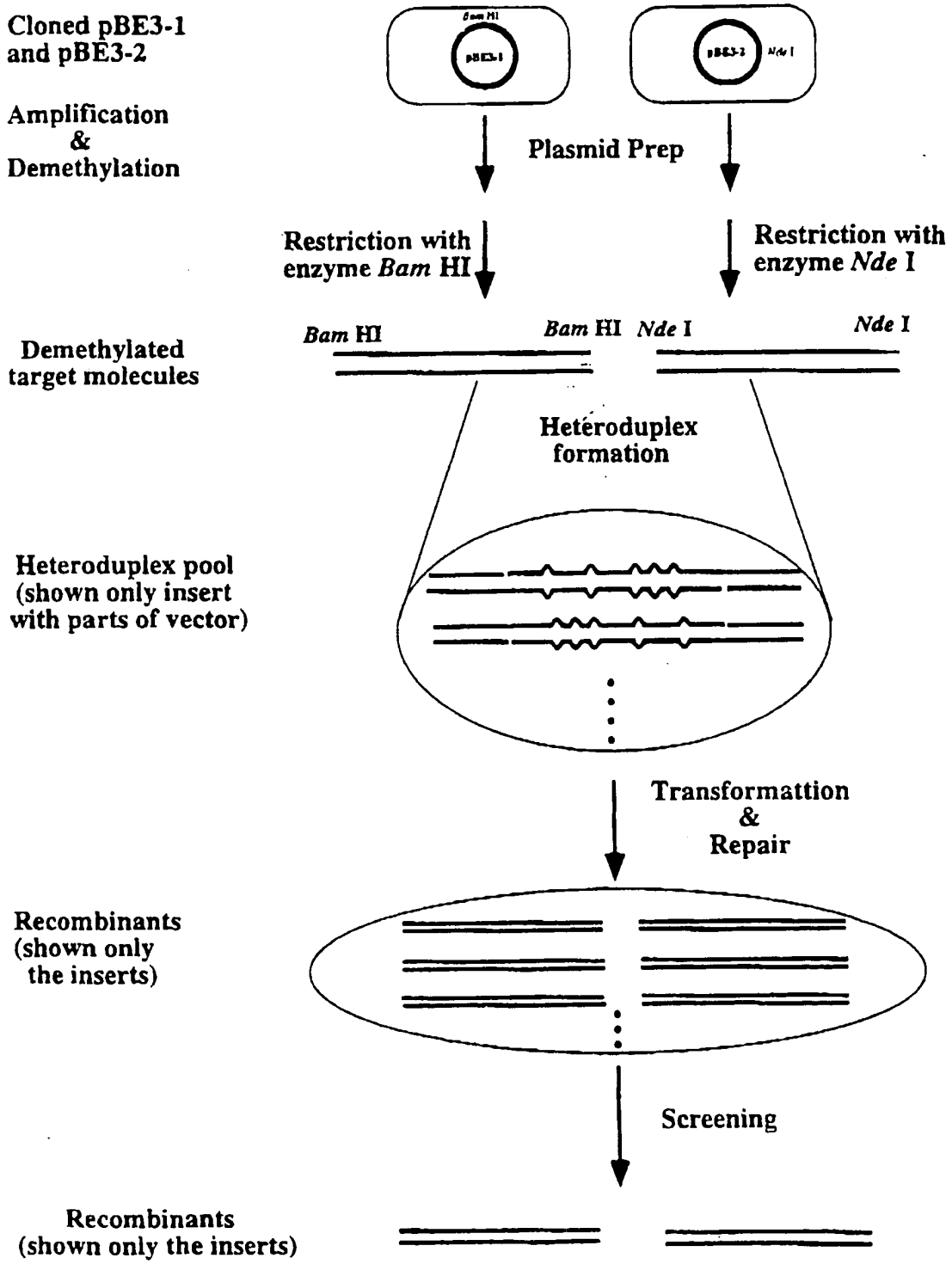
Cloned pBE3-1 and pBE3-2
Amplification & Demethylation
Plasmid Prep
Restriction with enzyme Bam HI
Restriction with enzyme Nde I
Demethylated target molecules
Bam HI
Bam HI
Nde I
Nde I
Heteroduplex formation
Heteroduplex pool (shown only insert with parts of vector)
Transformattion & Repair
Recombinants (shown only the inserts)
Screening
Recombinants (shown only the inserts)

Fig. 14

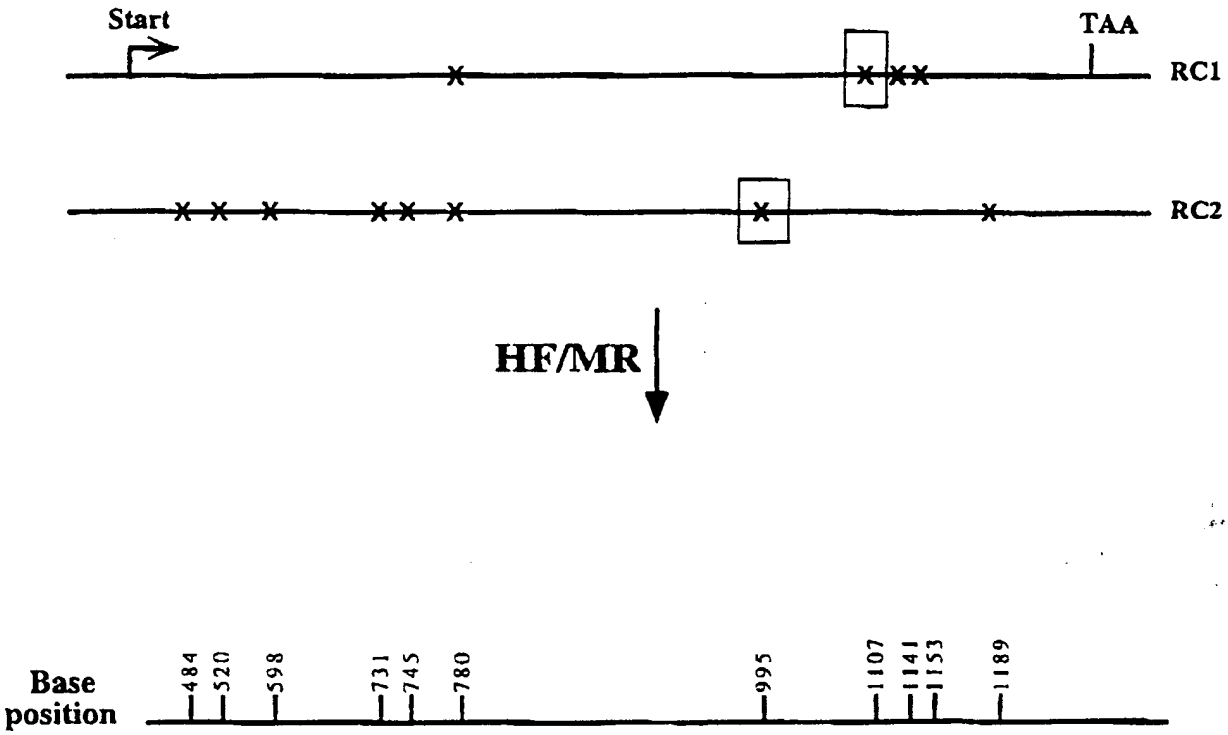
Start
TAA
RC1
RC2
HF/MR
Base position
484
520
598
731
745
780
995
1107
1141
1153
1189

Fig. 15

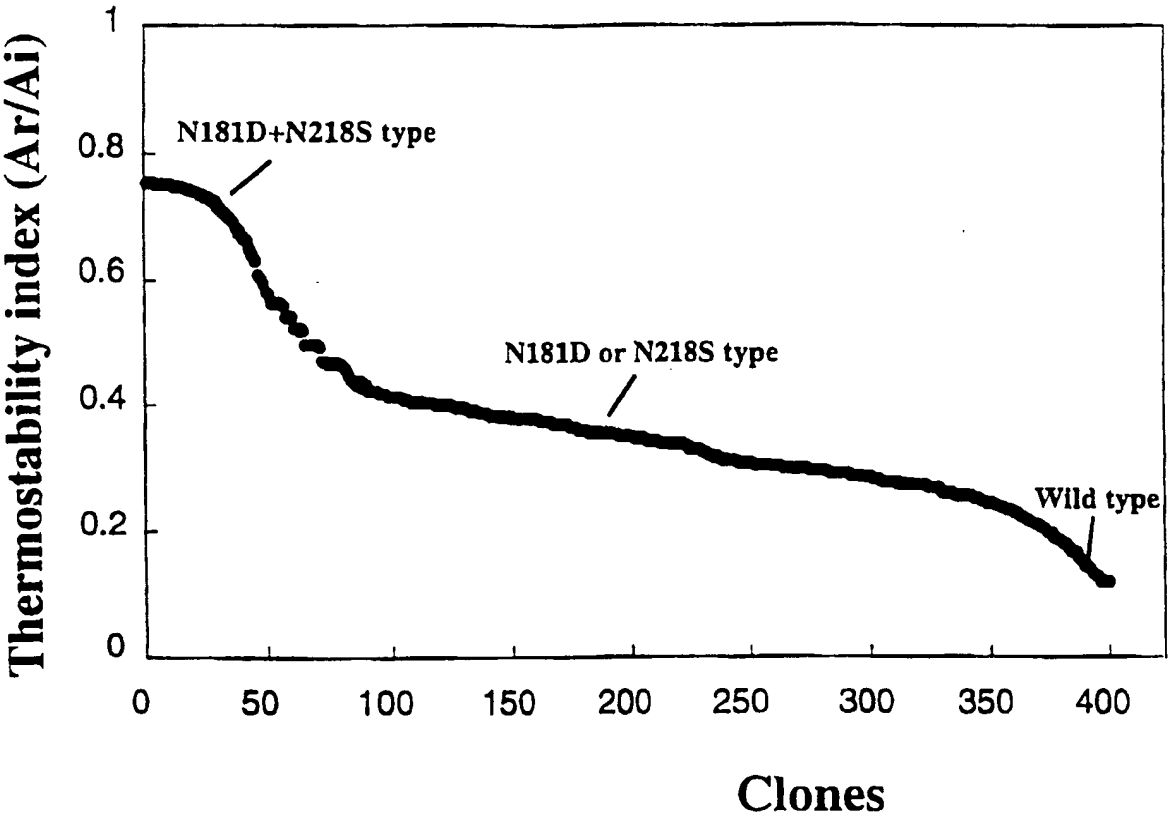
Thermostability index (Ar/Ai)
1
0.8
0.6
0.4
0.2
0
N181D+N218S type
N181D or N218S type
Wild type
0
50
100
150
200
250
300
350
400
Clones

Fig. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25698

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12Q 1/68; C12P 19/34
US CL :435/6, 91.1,91.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1,91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,679,522 A (MODRICH et al) 21 October 1997 (10/21/97), see entire document, especially abstract.	1-34
Y	FANG, W.H. et al. Human Strand-specific Mismatch Repair Occurs by a Bidirectional Mechanism Similar to That of the Bacterial Reaction. J. of Bio. Chem. June 1993, Vol. 268, No. 16, pages 11838-11844, see entire document.	1-34
Y	US 5,556,750 A (MODRICH et al) 17 September 1996 (09/17/96), see entire document especially abstract.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
26 MARCH 1999	26 APR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY SIEW Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25698

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS,STN,WPIDS,BIOSIS,MEDLINE,CANCERLIT,BIOTECHDS,LIFESCI,CAPLUS,EMBASE
search terms: amplification, heteroduplex, polynucleotides, repair,dna,pcr,vector